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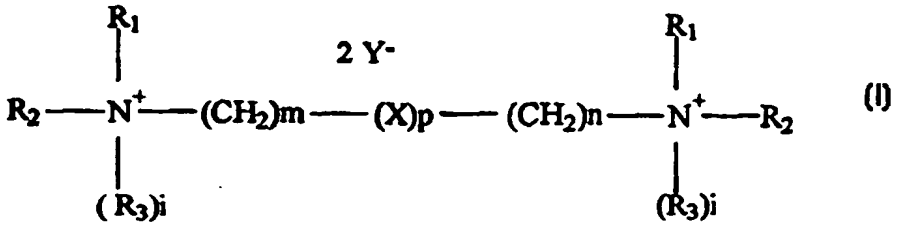
(54) Title: CATIONIC LIPIDS FOR TRANSFECTION OF NEGATIVELY CHARGED OR NEUTRAL MOLECULES INTO LIVING CELLS

(57) Abstract

A cationic lipid for transfection of nucleic acids comprising the mixture of a nucleic acid with a compound having structure (I), wherein Y = Cl-, Br-, I-, AcO-, or any pharmaceutically acceptable anion; X = O, S(O), CH_2; R_1, $R_{1'}$ = independently: C_1-C_{18} linear hydrocarbon; m, n = 1, 2, 3; R_2, $R_{2'}$ = independently: H; C_1-C_{18} linear alkyl; cyanoethyl; aminopropyl; aminobutyl; C_2-C_4 alkyl guanidinium or amidinium; N,N,N- independently (C_1-C_{18})aminopropyl or aminobutyl; C or N substituted spermine or spermidine; N,N-(C_4-C_{18})alkyl-4-aminonobutyrylaminopropyl; p = 0, 1; i = 0, 1; R_3, $R_{3'}$ = independently: C_1-C_6 linear alkyl, acetoxyethyl, $CH_2CO_2CH_2CH_3$; X ≠ CH_2 when R_2, $R_{2'}$ = C_1-C_6 linear alkyl; when p = 1, m and n ≠ 1.



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CATIONIC LIPIDS FOR TRANSFECTION OF NEGATIVELY CHARGED OR NEUTRAL MOLECULES INTO LIVING CELLS

Field of the Invention

This invention relates to polycationic lipids useful for the delivery (transfection) of nucleic acids (DNA,RNA) and other negatively charged or neutral molecules into living cells, either *in vivo* or *in vitro.*

Background of the Invention

Liposomes aggregates made with polycationic lipids are useful structures capable of complexing with negatively charged macromolecules such as DNA or RNA. These complexes can be taken up by living cells and then , once inside the cytosol, through an unknown mechanism, they are presumed to migrate into the cell nucleus. In the nucleus, there are enzymes capable of "reading" and "expressing" the message coded by the nucleic acids so delivered and produce new proteins, which were not being produced by the cell before the transfection of the foreign nucleic acid. When cells so transfected divide and their daughter cells still have the capability to produce the proteins encoded by the initially transfected DNA , the transfection is said to be stable. That is, the new DNA has stably integrated into the cell nucleus changing the cell's genetic make-up. If , on the other hand, the parent cells can produce the protein encoded by the transfected DNA, but their daughter cells are not capable of expressing the such DNA , the transfection is said to be transient. RNA transfection is always transient. Stable transfection of human or animal cells is the basis of the so called gene therapy, since cells which are deficient on a crucial protein for the organism's survival could be in principle repaired by stably transfecting the DNA needed to produce the absent protein. Another type of potential use of DNA/RNA transfection for therapy is the antisense therapy . In this approach, a short piece of nucleic acid (oligonucleotide) capable of adhering (hybridizing) to defective DNA (or RNA) which is being expressed by the cells to produce an undesired protein, such as an oncoprotein (cancer causing protein), is transfected into the cells in order to stop the expression of the undesired protein by virtue of its adherence to the defective nucleic acid. This method of therapy does not change the genetic make-up of the cell, but blocks the effect of the genetic disorder already present in the cell's genome. Besides this potential applications of polycationic lipids for use in human therapy, there is already a

well established market for these types of chemicals in the research products field. They are currently being used by researchers to deliver nucleic acids and proteins into cells in order to study how the expression of different genes affect cell growth and function.

There are two possible ways to deliver DNA into cells for gene therapy : *ex vivo* and *in vivo* transfection. In the *ex vivo* modality , cells from a patient are removed from the body, cultured and transfected *in vitro*. Then, the cells are returned into the patient where the beneficial DNA message is hopefully expressed. In the *in vivo* mode, the DNA is delivered directly into the patient, which makes this procedure simpler and less expensive. To date the only effective way to deliver DNA *in vivo* is by using a virus which naturally infects cells of an specific organ (targets that organ) within the body, and whose genetic make up has been modified by adding the DNA beneficial to the patient. Once inside the cells of the patient, the virus can incorporate the new DNA in the genome of the cell (stable transfection) and the parent cell and its daughters can express the beneficial protein. The pathological component of the virus has been deleted before the patient is exposed to such a virus and only the targeting component left intact. Virus can do this process sometimes with nearly 100% efficiency. However, there are risks associated with their use, they can produce immunological reactions which may be fatal to the patient; the DNA incorporation in the cell's genome is random, therefore it might disrupt needed genes or activate oncogenes; they are also difficult to mass produce, etc.

Liposomes or lipid aggregates do not have the side effects of viruses , but are not as efficient as viruses are. There is a constant need to develop newer lipids that can approach the efficiency of viruses without their undesirable side effects (E. Marshall, *Science* 269,1050 (1995)) . There are several lipids for nucleic acids transfection already in the market. The most relevant of these lipids are: DOTMA (N-[1-(2,3-dioleoyloxy)propyl]- N,N,N-trimethylamonium chloride, U.S. Pat. No. 4,897,355 to D. Eppstein et al.), DMRIE (D,L-1,2-O-dimyristyl-3-dimethylaminopropyl-b-hydroxyethylammoniumchloride, U.S. pat. No 5,264,618 to Felgner, P.L. et al.), DOTAP (1,2-bis(oleoyloxy)-3-3(trimethylammonia)propane) Boehringer-Mannheim Catalog No.1 202 375) , DOGS (5-carboxysperminyglycine dioctadecylamide, U.S. pat. No 5,171,678 to Behr, J-P. et al. DOGS is sold under the trade name Transfectam™ by the Promega

Corp. Madison, WI), DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxyamido)ethyl]-N,N-dimethyl-propanaminium trifluoroacetate, U.S. Pat. No. 5,334,761 to Gebeyehu, G. et al.), DDAB (Dimethyloctadecylammoniumbromide, U.S. Pat. No. 5,279,833 to Rose, J.K.), TMTPS (N,N,N,N-Tetramethyltetrapalmylspermine, PCT Int.Pub.No. WO 95/17373. Haces, A. et al.). DOTMA, DOSPA, DDAB and TMTPS are sold by Life Technologies, Inc., Gaithersburg, MD under the trade names of Lipofectin, LipofectAMINE, LipofectACE and CellFECTIN, respectively. A recent relevant publication which deals with art related to the present invention has been reported by Ruysschaert et al.((1994)*Biochem. Biophys. Res. Commun.*203,1622-1228). All these lipids, except DOGS, are formulated with dioleolylphosphatidylethanolamine (DOPE), which is a neutral lipid devoid of transfection activity, in order to make the active liposomes. These lipids posses some desirable characteristics, however they are far from the ideal vehicle to deliver DNA. Their main drawbacks are low efficiency, non-specificity of targeting, considerably toxicity, low water solubility, and serum inhibition of their action.

Although progress has been made in overcoming some of these obstacles, there is considerable room for improvement an experimentation. The design of these lipids is still a semi-empirical endeavor , since very little is known about the mechanism by which they act.

Therefore, it is the object of this invention to improve the desired characteristics of these lipids by exploring and incorporating new chemical functionalities as well as spatial or topological arrangements which improve the transfection efficiency and lower the toxicity.

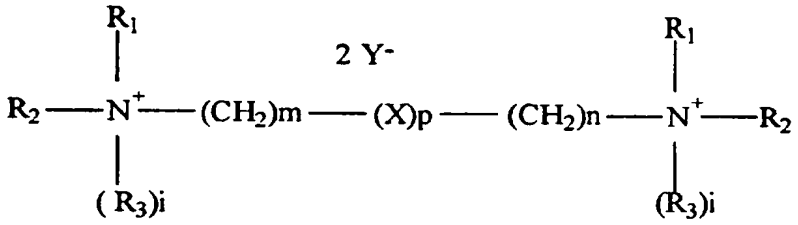
It is also an object of this invention to synthesize polycationic lipids which incorporate a small , non lipid-bilayer-disturbing moiety that mimics a natural molecule, which cells can recognize as their natural effector or ligand, thus facilitating the transfection as well as the specificity of targeting of the macromolecule.

Summary of the Invention

In this invention a series of new polycationic lipids and their method of preparation is described Such lipids are useful as transfection reagents for: nucleic acids, oligonucleotides,mononucleotides, polypeptides and proteins. In addition, some of these lipids are also useful as more effective detergents for cleaning and as vehicles in the cosmetic field.

The present invention describes novel oxo and sulfinyl backbone substituted polycationic lipids with ammonium, guanidinium and imidinium positively charges moieties as anchoring groups having the formula:

Formula I



Y= Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion

X = O,S(O),CH_2 R_1,R_1' = independently: C_1-C_{18} linear hydrocarbon.

m,n = 1,2,3 R_2,R_2' = independently : H ; C_1-C_{18} linear alkyl ; cyanoethyl; aminopropyl ; aminobutyl; C_2 -C_4 alkyl guanidinium or amidinium;N,N,N independently(C_1C_{18})aminopropyl or butyl; C or N substituted spermine or spermidine; N,N-(C_4-C_{18})alkyl-4- aminonobutyrylaminopropyl

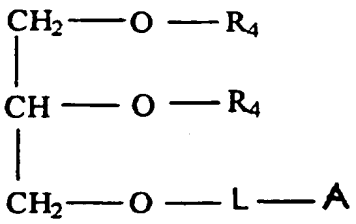
p = 0, 1 R_3,R_3' = independently: C_1-C_6 linear alkyl, acetoxyethyl,
i = 0, 1 $CH_2CO_2CH_2CH_3$

$X \neq CH_2$ when R_2,R_2' = C_1-C_6 linear alkyl

When p = 1, m and n ≠ 1

It is also disclosed in this invention a series of novel phosphatidyl and glyceryl guanidinium cationic lipids having the formula II.

Formula II



R_4 = Independently: linear $(CO)C_6$-C_{20}. C_6-C_{20}

L = 2-10 atoms linker

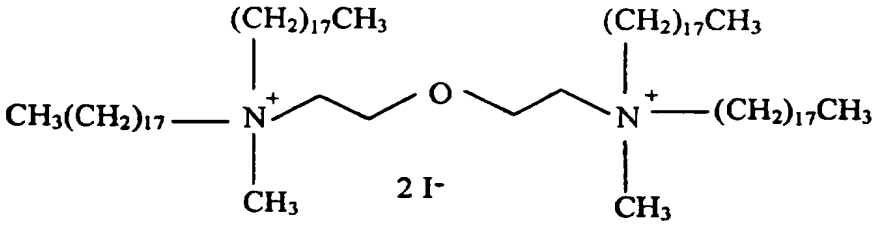
A = Guanidinium; imidinium; guanidylated polyamines.

These compounds can be used alone or in mixtures with other liposome forming compounds (co-lipids) to prepare lipid aggregates which are useful to deliver macromolecules, specifically negatively charged macromolecules to living cells either in culture or *in vivo*.

Compounds of Formula I:

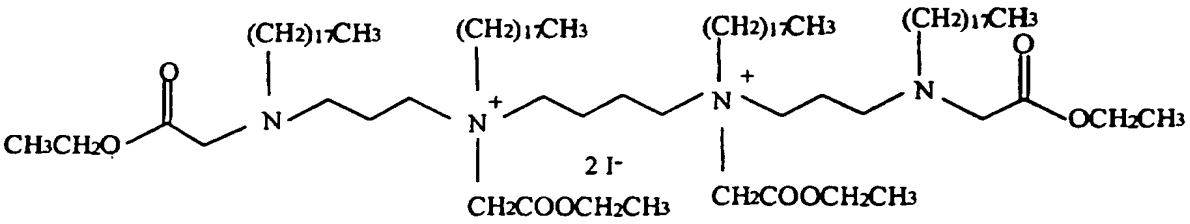
The lipids depicted in Formula I have a hydrocarbon backbone substituted with heteroatoms which are sterically smaller, but equally or more flexible as the methylene group that they replace. This feature makes these new lipids fit more closely to the macromolecule to be delivered to the cells. This closer fit combined with the polycationic nature of the backbone produces a tighter binding. In addition, these heteroatoms are hydrophilic; thus, they not only confer an increased amphiphilic character to the lipids but also make the backbone more linear or "stretched" as compared to the all-methylene groups backbone. The latter being hydrophobic tends to wrap around itself in an aqueous environment, therefore pulling the positively charged moieties away from the negatively charged phosphates on the DNA/RNA backbone, this results on a weaker binding between the polycationic lipid backbone and the polyanionic DNA backbone, since the opposite charges can not align properly in this arrangement. The hydrophilic backbone being linear allows for proper alignment of the opposite charges, also leading to a tighter

binding. In addition, the higher hydrophilicity conferred by the heteroatoms on the polycationic backbone, make possible the addition of more hydrophobic tails without loss of water solubility, thus making these compounds more densely packed than compounds of the prior art. This is an advantage since the same molar amount of lipid will produce a higher hydrophobic coating of the nucleic acid to be delivered. In fact, one of the preferred embodiments is compound (5a) which has four hydrophobic tails and two positive charges (two tails per charge).

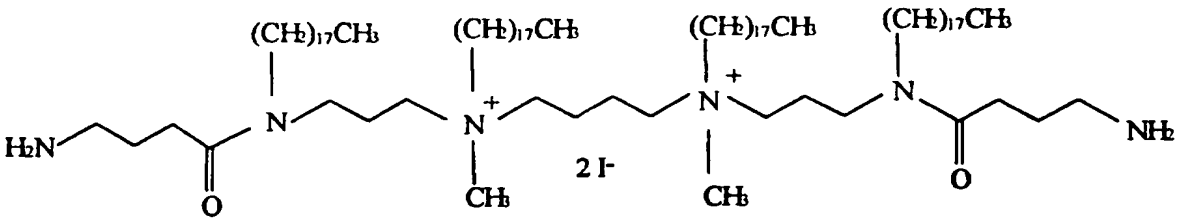


(5a)

Some of the compounds herein described, such as (13a,b) have fewer hydrophobic tails and a more hydrophobic backbone (no heteroatom substitution, but less hydrophobic overall). However, these compounds have moieties which mimic small natural biological effectors such as the neurotransmitters gamma amino butyric acid (GABA), acetylcholine etc. These moieties bind to their corresponding cell receptors targeting the delivery of nucleic acids to those cells rich in these type of receptors such as muscle and neural cells. The latter type of cells are among the most difficult to transfect since they are postmitotic cells (non-dividing). These small chemical moieties do not perturb the ability of the lipids to form liposomes aggregates and at the same time confer more amphiphilic character to said lipids, since they are polar entities. A particular preferred embodiment of the latter compounds are compounds of formulae (13a) and more specifically compound (13d).



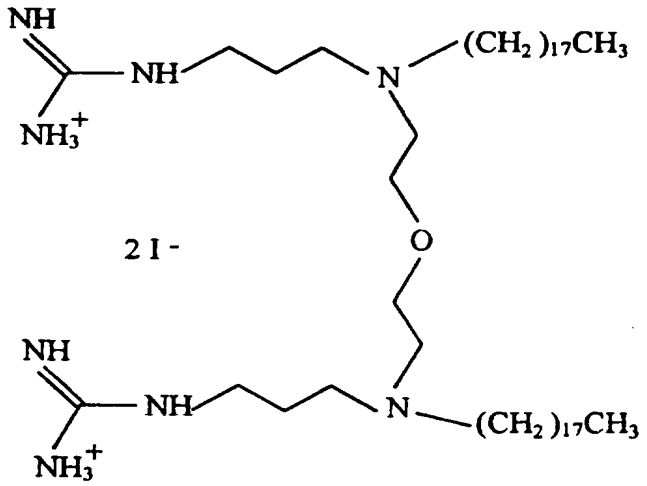
(13a)



(13d)

Another novel feature of the compounds disclosed here is the fact that, in addition to the traditional quaternary ammonium salts, guanidino and amidino moieties are used as permanent positively charged centers. These functional groups are strongly basic an have the same charge as their ammonium counterparts, but have the advantage of being sterically smaller , since they are planar. Thus, they can get closer to the negatively charged phosphates of the DNA/ RNA backbone producing a stronger binding interaction than that of ammonium salts. Furthermore, these guanidinium and amidinium moieties have the ability to form hydrogen bridges with the nucleic acids bases (guanidinium salts are used as chaotropic agents to precipitate DNA) therefore they have an additional binding mode not available to ammonium salts. Moreover, the guanidino moiety can also be used to target neural cells, since compounds such as Guanethidine , which possess such a functional group, are internalized by neurons (Wiener, N. In, *The Pharmacological Basis of Therapeutics*, (Gilman, A.G.; Goodman,L.S.; Rall, T.W.; and Murad, F.; Eds.) Macmillan Pubs. Co. New York, 1985,pp. 181-214.) . Thus , by including the lipidic content as well as the amine and guanidino moieties of Guanethidine in our novel

liposome reagents we can target this difficult to transfect cell type. A particularly useful and preferred embodiment of these compounds is compound (6d), which is the most active of compounds tested.

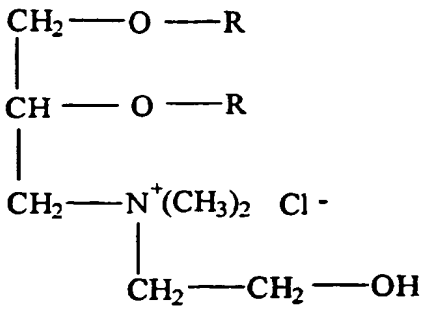


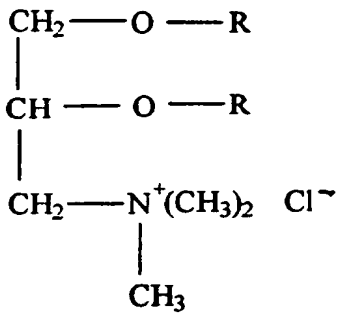
(6d)

Additionally, reduced or no toxicity was observed for these lipids at the concentrations tested.

Compounds of Formula II:

Despite all the reasons given above in order to "rationally design" these lipids, it is still impossible to predict their DNA transfection activity at this time. In fact, cationic lipids for DNA transfection already in the market such DOTAP, DOTMA, DMRIE and DORI whose chemical structures are almost identical to that of the cationic lipid known as the Rosenthal Inhibitor. (Rosenthal, A.F. and Geyer, R.P., *J. Biol. Chem.* 235(8):2202 (1960)) have significant transfection activity, unlike the Rosenthal Inhibitor which is reported to be inactive as a DNA transfection reagent (see U.S. pat No 5,264,618 to Felgner, P.L. et al.). The following formulae illustrate this point more clearly :



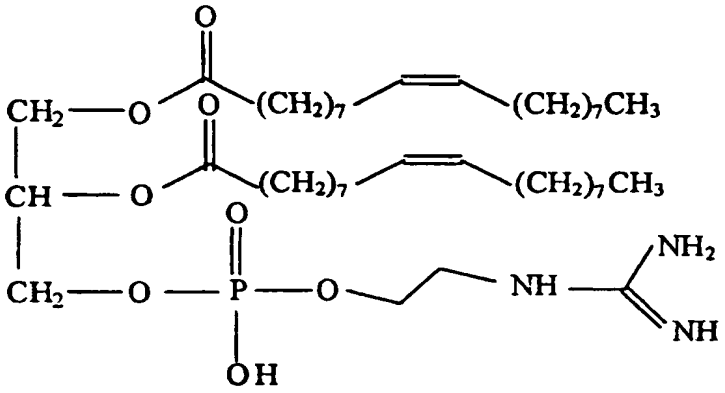


R = Stearoyl, Rosenthal inhibitor (inactive)
R = Myristyl, DMRIE (active)
R = Oleoyl, DORI diester (active)

R = Oleyl, DOTMA (active)
R = Oleoyl, DOTAP (active)

Compounds of formula II described herein have also a similar structure to that of the Rosenthal Inhibitor. However, these compounds differ from the Rosenthal Inhibitor on that a guanidinium or amidinium functionality is used as the positively charged anchoring group, and they also lack a quaternary ammonium group at the C-1 position of the glycerol backbone. A preferred embodiment of these latter type of transfection reagents is compound (15).

(15)



An interesting feature of this compound is that it has the ability to form liposomes without the need of co-adjuvants such as DOPE or DOPC. Thus, it can be used to form liposomes with other cationic lipid compounds.

Specific Examples of the Invention.

Scheme I

This reactions scheme shows the general synthetic route to prepare polycationic lipids having a heteroatom substituted anchoring backbone. Thus, diglycolyl chloride (1) is treated with a suitable primary or secondary amine (2a-d) in methylene chloride in the presence of a base such as triethyl amine under an inert gas such as argon at room temperature to obtain the corresponding diglycolamides (3a-d). These amides can then be reduced with lithium aluminum hydride or borane in refluxing anhydrous tetrahydrofurane (THF) to afford the corresponding amines (4a-d). Secondary amine (4c) was easily converted to the corresponding tertiary amine (4e) upon treatment with acrylonitrile. Compound (4e) can be treated with ammonium chloride at high temperature to produce the corresponding amidine(6e). Alternatively, the latter amidine derivatives can be obtained by reacting the dinitrile (4e) with anhydrous hydrogen chloride in ethanol, followed of treatment of the imidoester so obtained with ammonium hydroxide. Primary amine (4d) can be converted to the target compound (6) by treatment with S-methyl isothiouronium hydroiodide (S-methyl thiourea) in tetrahydrofurane in the presence of triethylamine. Additionally , this guanidinium derivative can be alkylated with for example iodo methane to produce the corresponding quaternary ammonium salt. Tertiary amines (4a,b,e) are treated with an alkylating agent such as iodo methane , iodoethyl acetate or 2- bromo ethyl acetate to afford the quaternary ammonium salts (5a,b,f). The latter compounds were also synthesized by treating the corresponding tertiary amines with the commercially available 2-bromoethyl ether (lower panel, scheme I). This route has only two steps , but is not as flexible or prolific as the route depicted on scheme I.

Scheme II

Compound (7) is easily synthesized by treating commercially available 1,4-diaminobutane with acrylonitrile. Diamide-dinitrile (8) is then easily obtained by treatment of compound (7) with an acyl halide such as palmitoyl chloride in methylene chloride in the presence of triethyl amine. The diamide-dinitrile (8) can be reduced with lithium aluminum hydride or borane in THF to the corresponding tertiary and primary amines

functionalities to afford compound (9). Guanidinium compound (10) can be obtained in a similar fashion as shown in scheme I for compound (6d) by reacting the primary amines of compound (9) with S-methyl thiourea in THF and triethylamine.

Scheme III

Tetrapalmyl spermine (11) (Haces, A. et al. PCT Int. Pub. No WO 95/17373) was treated with ethyl iodoacetate at room temperature to afford the tetraalkylated derivative (13a). Similarly, compound (11) can be treated with 2-bromoethyl acetate at high temperature to afford (13b). Reaction of (11) with 4-bromo or 4- chloro butyryl chloride in methylene chloride in the presence of triethyl amine at low temperature gives the corresponding 4-bromobutyramide derivative (12). Intermediate (12) is immediately treated with iodo methane to produce the N,'N"- dimethylated intermediate (13c) which in turn is treated with an excess of ammonium hydroxide in tetrahydrofurane at elevated temperature to convert the bromide (or chloride) into the corresponding primary amine (13d). All these compounds have moieties which resemble or mimic the neurotransmitters gamma aminobutyric acid (GABA, compound (13d)) and acetyl choline (compounds (13a,b)). These small groups do not change substantially the liposomal forming ability of the lipid molecule and at the same time are capable of being recognized by neural or muscles cells. This preferential recognition by these type of cells makes these lipids target specific DNA/RNA delivery agents.

Scheme IV

Commercially available dioleoylphosphatidylethanolamine (14) was treated with an excess of S-methyl isothiouronium hydroiodide in tetrahydrofurane and in the presence of triethylamine to afford the corresponding guanidinium compound (15).

EXAMPLES

Example 1: Synthesis of octadecylcyanoethylamine (2d).

Octadecylamine (2g, 7.4 mmol) and acrylonitrile (15ml) were heated for 3h at 70°C in a thick wall test tube capped with a teflon lined cap. TLC (silica gel; ethyl acetate) shows a new spot at rf = 0.65 . The excess acrylonitrile was removed in vacuo to afford pure product (2.40g, 100% yield). H-NMR ($CDCl_3$) δ: 0.88 (t, 3H), 2.25 (br.s.,32H), 2.53 (t, 2H), 2.62 (t,2H), 2.93 (t,2H). FT-IR (cm^{-1}) 2250 (CN).

Example 2: Synthesis of bis (mono and dialkyl)diglycolamides(3a-d), general procedure.

To a solution of dialkylamine (2mmol) and triethylamine (2 mmol) in methylene chloride (250ml) under argon was added diglycolyl chloride (1mmol) and the resulting solution was stirred for 18h at room temperature. TLC (silica gel; MeOH or CH_2CL_2/THF,3:1) shows absence of starting material and a new spot. The methylene chloride solution was washed with sodium bicarbonate (10% in water), dried (Na_2SO_4) and the solvent removed, to afford the desired diamide.

Proceeding as described before and using the appropriate mono or dialkylmine the following compounds were prepared:

3a. Bis (dioctadecyl) diglycolamide (81% yield), H-NMR (CDCL3) δ: 0.88 (t,12H), 1.25 (s, 124H), 1.5 (br.s, 8H), 3.15(t,4H), 2.9 (t,4H), 3.2 (s, 4H). R (cm^{-1}): 1651 (C=O);

3b. Bis (didecyl)diglycolamide (73% yield), H-NMR(CDCL3)d X: 0.85 (t,12H), 1.25 (s, 64H), 1.5 (br.s,8H), 3.18 (t,4H), 3.3 (t,4H), 4.4 (s, 4H). FT-IR(cm^{-1}): 1657(C=O);

3c. Bis (octadecyl)diglycolamide (100% yield),H- NMR(CDCL3) δ: 0.85 (t, 6H), 1.25 (br.s, 60H), 1.5 (br.s,4H), 3.3 (q,4H), 4.05(s,4H), 6.4 (br.s,2H);

3d. Bis (octadecylcyanoethyl) diglycolamide (98%yield), H-NMR ($CDCl_3$) δ: 0.85 (br.t, 6H), 1.1-1.6 (br.s, 64H), 2.65 (t, 4H), 3.25 (br.t, 4H), 3.55 (br.t, 4H), 4.3 (s, 4H);

Example 3: Synthesis of N,N,N',N'_-Mono and dialkyl 2,2'-oxybis ethylamines (4a-d), general procedure

To a solution of lithium aluminum hydride (6 to 64 molar excess) in dry tetrahydrofurane (THF) was added the corresponding diamide in small portions under a blanket of argon. The resulting mixture was refluxed for two to three days under argon. The progress of the reaction was followed by TLC (silica gel; CH_2Cl_2/TFH, 3:1 for dialkylamides; 10% triethylamine in CH_2Cl_2 for monoalkylamides and triethylamine for cyanoethylamides). The reactions were quenched with sodium hydroxide (10% in water). The mixture was filtered, the filtrate dried (Na_2SO_4), and the solvent removed in vacuo to afford the desired products.

Proceeding as described above and using the appropriate diamide the following compounds were obtained:

4a. N,N,N',N'-dioctadecyl-2,2'-oxobis ethylamine (77% yield), H-NMR($CDCl_3$) δ: 0.88 (t, 12H), 1.25 and 1.43 (br.s., 128H), 2.42 (t, 4H), 2.62 (t, 4H), 3.48 (t, 4H), 3.7, (t,4H);

4b. N,N,N',N'-didecyl-2,2'-oxobis ethylamine (93% yield), H-NMR ($CDCL_3$) δ: 0.88 (t,12H), 1.25 and 1.42 (br.s, 64H), 2.42 (t, 8H), 2.63 (t, 4H), 3.5 (t, 4H);

4c. N,N',-octadecyl-2,2'-oxobis ethylamine (71% yield), H-NMR ($CDCl_3$) δ: 0.87 (t, 6H), 1.25 and 1.45 (br.s., 64H), 2.6 (t,4H), 2.78 (t, 4H), 3.65 (t,4H);

4d. N,N,N',N'-octadecylaminopropyl-2,2'-oxobis ethylamine (80% yield), H-NMR (CDCL3) δ:0.88 (t,6H), 1.25 and 1.45 (br.s,70H), 2.4-2.8 (br.m,16H), 3.4-3.7 (br.m, 4H).

Example 4: Synthesis of N,N,N,N',N',N'_- dioctadecylmethyl-2,2'-oxybisethylammonium iodide.(5a).

N,N,N',N'-dioctadecyl-2,2'-oxy bis ethylamine (19mg, 0.017mmol) was dissolved in iodo methane (1ml) inside a capped thick-wall test tube, and the resulting solution heated for 20h at 75°C. TLC (silicagel; chloroform:acetone:methanol:water; 50:15:5:5:1) shows only one spot at Rf = 0.8 ,which gives a negative ninhydrin test and no starting material. The excess iodo methane was removed in vacuo to afford desired product (23 mg, 96%). H-NMR($CDCl_3$) δ: 0.88 (t,12H), 1.15 - 1.5 and1.7 (br.s.,128H), 3.35 (s, 6H),

3.46 (br.m.,8H), 3.88 (br.s.,4H), 4.28 (br.s., 4H). Proceeding in a similar fashion as per compound (5a) , compound (5b) was obtained in 100% yield.

Example 5: Synthesis of N,N,N',N'-cyanoethyloctadecyl-2,2'-oxybis ethylamine (4e).

A suspension of N,N'-octadecyl-2,2'-oxybis ethylamine (100mg,0.16mmol) in acrylonitrile (4ml) was heated for 18h at 80°C in a capped, thick-wall test tube (the initially liquid two phase system became a clear homogenous solution after 2h). TLC (silicagel ; dichloromethane / THF, 3:1) shows absence of starting material and a spot corresponding to desired material at rf = 0.95. The excess acrylonitrile was removed in vacuo to afford desired material. H-NMR($CDCl_3$) δ: 0.88 (t, 6H), 1.25 (br.s., 64H), 2.45 (2t,8H), 2.68 (t, 4H), 2.87 (t,4H), 3.5 (t, 4H).

Example 6: Synthesis of N,N,N,N',N',N'-cyanoethyloctadecylmethyl-2,2'-oxybis ethyl ammonium iodide (5e).

A solution of N,N,N',N'-cyanoethyloctadecyl-2,2'-oxybis ethylamine (60 mg,0.08 mmol) in iodo methane (1.5ml) was heated for 3h at 80°C in a capped, thick wall tested tube. The excess iodo methane was removed in vacuo to afford desired product. H-NMR ($CDCl_3$) δ: 0.88 (t, 6H), 1.25 (br.S., 60H), 1.8 (br.s., 4H), 3.45 (br.s., 6H), 3.65 (br.s., 4H), 3.95-4.4 (br.m., 16H).

Example 7: Synthesis of N,N,N,N',N',N'- acetoxyethyldioctadecyl-2,2'-oxybis ethyl ammonium iodide(5f).

A solution of N,N,N',N'-dioctadecyl-2,2'- oxybis ethylamine (16mg, 14.3 mmol) and ethyl iodoacetate (0.5ml) in chloroform (1ml) was heated for 18h at 75 °C in a capped thick wall test tube. The chloroform was removed in vacuo and the residue dissolved in tetrahydrofurane (10ml). To this solution was added thiourea and the mixture stirred at room temperature until no more thiourea went into solution (stoichiometric excess after all iodide is converted to the isothiouronium salt). The mixture was heated for 2h at 70°C and the excess solvent removed in vacuo. The residue was then redissolved in

dichloromethane (10ml) and the solution washed with water (4x 5 ml), dried (Na_2SO_4) , filtered and the solvent removed in vacuo to afford 10mg of desired product.

Example 8: Synthesis of S-methylisothiouronium hydroiodide.

A solution of thiourea (0.8g,10.5mmol) and iodo methane (8.8g,62mmol) in methanol (25ml) was heated in a capped thick-wall test tube for 4.5h at 50°C. The reaction mixture was rotaevaporated to afford pure desired material in 100% yield.H-NMR (CD_3OD) δ: 2.62(s, 3H), 4.8 (br.s., 4H).

Example 9: Synthesis of N,N,N',N'-guanidinopropyloctadecyl oxy bis-2,2'-ethylamine hydroiodide (6d).

A solution of N,N,N',N'-aminopropyloctadecyl-2,2'- oxybis ethylamine (100mg, 0.14 mmol) , S- methyl isothiouronium hydroiodide (300mg, 1.3 mmol) and triethylamine (300mg, 3 mmol) in tetrahydrofurane (10ml) were heated in an argon flushed, capped thick-wall test tube for 20h at 95°C. The solvent and methyl mercaptan by product were removed in vacuo in a chemical fumes hood and the residue dissolved in methylene chloride (30ml), the organic phase was washed with brine (3x, 20 ml), water (2x 10ml), dried (Na_2SO_4), filtered and the solvent removed to obtain a reddish solid (100mg,80%). H-NMR($CDCl_3$) δ: 0.86 (t,6H), 1.1-1.6 (br.s., 64H), 2.4-2.8 (br.m., 16H), 3.15-3.7 (br.m., 12H). FTIR (cm^{-1}): 1653 (C=NH).

Example 10: Synthesis of N,N'-cyanoethyl-1,4-diaminobutane (7).

1,4-diaminobutane (2g, 22 mmol) was cooled to 0C° (ice bath) and to this solid was added acrylonitrile (4ml) . The mixture was let reach room temperature slowly (ca 30min) and then let react for additional 18h at room temperature with stirring. The excess acrylonitrile was removed in vacuo to afford the desired product. H-NMR ($CDCl_3$) δ: 1.3 (br.s.,2H), 1.5 (br.s, 4H), 2.5 (t, 4H), 2.65 (br.m.,4H), 3.9 (t,4H). FTIR (cm^{-1}) : 2247 (C=N).

Example 11: Synthesis of N,N,N',N'-cyanoethylpamitoyl-1,4-diaminobutane(8).

To a solution of N,N'-cyanoethyl-1,4-diaminobutane (0.714g , 3.68mmol) and triethylamine (0.744g ,7.36mmol) in dichloromethane (150ml) was slowly added palmitoyl chloride (2.02g, 7.36 mmol) and the resulting mixture let react at room temperature overnight. The reaction was washed with sodium bicarbonate (10%,2x 50ml), water (2x 50ml), dried (Na_2SO_4) , filtered and the solvent removed in vacuo to afford the desired product (2.3g, 93 %). H-NMR ($CDCL_3$) δ: 1.9 (t,6H), 1.25 and 1.6 (br.s., 56H), 2.32 (m,4H), 2.72 (t, 4H), 3.42 (m, 4H), 3.55 (t,4H). FTIR (cm^{-1}) 1643 (C=O).

Example 12: Synthesis of N,N,N',N'-aminopropylpalmyl-1,4-diaminobutane (9).

To a solution of lithium aluminum hydride (600mg,15.9 mmol) in tetrahydrofurane (50ml) was added N,N,N',N'-cyanoethylpalmitoyl-1,4-diaminobutane (300mg, 0.45 mmol) and the reaction mixture was refluxed for 72 h. Then, a procedure essentially the same as per example 3 (supra) was followed to afford desired diamine (200mg,70%). H-NMR($CDCl_3$) δ: 0.88 (t,6H), 1.25 and 1.5 (br.s, 64H), 2.3-2.7 (br.m, overlap. t, 16H).

Example 13: Synthesis of N,N,N',N'-guanidinopropylpalmyl-1,4-diaminobutane (10).

A procedure identical as per example 6 (supra) was followed. H-NMR ($CDCl_3$) δ: 0.9 (t, 6H), 1.2-1.35 (br.s., 68H), 1.5-2.0 (br.m., 4H), 2.65-3.5 (m, 24H). FTIR (cm^{-1}) : 1650 (C=N).

Example 14: Synthesis of N,N'''-4-bromobutyryl-N,N',N''N'''-tetrapamylspermine (12).

To a cooled (0 ^{0}C) solution of N,N',N'',N'''-tetrapalmylspermine (400mg, 0.36 mmol) and triethylamine (80mg, 0.8 mmol) in dichloromethane (14ml) was added 4-bromobutyryl chloride (156mg, 0.8 mmol) and the resulting mixture let react for 1h at 0 ^{o}C. The reaction was quenched and washed with cold sodium bicarbonate solution (10 % in water,3x 5ml), dried (sodium sulfate), filtered and the solvent removed at room temperature in vacuo to afford a white foam.. H-NMR ($CDCl_3$) δ: 0.88 (t, 12H), 1.15-1.5 (br.s., 136H), 2.12 (t, 4H), 2.3-2.55 (br.m.,12H), 3.2-3.36 (br.m., 8H), 3.62 (t, 4H).

Example 15: Synthesis of N,N',N",N"'-tetrapalmyltetraacetoxyethylspermine iodide salt(13a):

A solution of tetrapalmylspermine (Haces et al.,PCT Int. Pub. No WO/95/17373), 130mg, mmol) in neat ethyl iodoacetate (1.5ml) was heated to 75 °C for 18h. The reaction was worked up following essentially the same procedure as per example 13 (supra) to afford the desired product.

Example 16: Synthesis of N,N"'-4-bromobutyryl-N,N',N",N"'-tetrapalmyl-N',N"-dimethylspermine(13c).

N,N"' - 4-bromobutyryl-N,N',N",N"'-tetrapalmylspermine (350mg,0.25 mmol) was dissolved in iodo methane (3ml) and the resulting solution let react for 2 days at room temperature . Excess iodo methane was evaporated to afford desired product, which is negative for ninhydrin test. H-NMR ($CDCl_3$) δ: 0.88 (t, 12H), 3.41 (br.s., 6H).

Example 17: Synthesis of N,N"'-4-aminobutyryl-N,N',N",N"'-tetrapalmyl-N',N"-dimethylspermine(13d).

To a solution of N,N"'-4-bromobutyryl-N,N',N",N"'-tetrapalmyl-N',N"-dimethylspermine (100mg,0.05 mmol) in tetrahydrofurane (10ml) was added ammonium hydroxide (20 ml, 28% by weight) and the resulting mixture heated to 70 °C for 2 days in a capped, thick wall reaction tube. The solvent was azeotropically evaporated (ethanol) to afford a brown solid which is strongly positive for ninhydrin test.H-NMR ($CDCl_3$) δ: 0.88 (t,12H), 1.1-1.4 (br.s,130H),1.5-2.2 (br.m,16H), 3.0-3.8 (br.m,10H), 3.4 (br.s, 6H).

Example 18: Synthesis of dioleoylphosphatidyl ethanolguanidine (15).

To a solution of dioleolylphosphatidyl ethanoamine (70mg,0.094 mmol) and triethylamine (1ml) in tetrahydrofurane (10 ml) was added S-methylisothiouronium hydroiodide (70mg, 0.32 mmol) and the resolting solution heated for 18h at 70 °C. The solvent was removed in vacuo and the residue redissolved in dichloromethane (25ml). This solution was washed with water (2 x 10 ml), dried (sodium sulfate), filtered and the solvent evaporated to afford the desired product (40 mg, 47%). H-NMR ($CDCl_3$) δ: 0.88

(t,6H), 1.2-1.44 (br.s, 40H), 2.00 (br.d,10H), 2.29 (t, 4H), 3.19 (q,1H), 3.42 (br.s,1H), 3.85-4.20 (br.m,2H), 4.38 (br.m,1H), 5.3 (br.d, 4H). FTIR (cm^{-1}) : 2361, 1741.

Example 19: Liposomes formulation:

Lipids were formulated by mixing the appropriated molar amounts of the active lipid with dioleoylphophatidyl ethanolamine (DOPE) in dichloromethane and dispersing this mixture in the final amount of water using the solvent vaporization method. (David W. Deamer, in Liposome Technology, vol.I, p-29, CRC press Boca Raton, Fl, 1984).

Cell culture and plasmids

Cell lines were from the American Type Culture Collection (Rockville, Maryland) and were cultured in RPMI1649, 10% FCS, pen/strep . Plasmid pCMV β-gal, which contains the *E. Coli* β-galactosidase (gene) under the control of the powerful cytomegalovirus promoter (McGregor et al. (1989) *Nucleic Acids Res.*, 17.:2365) was purchased from Clontech, Inc. Primary cells were from human tracheal isolates and neonatal foreskin.

Example 20: Transfection of HepG2 and HeLa cells.

Cells were plated in 48-well plates ($1cm^2$) at a concentration of $1x.10^5$ cells/well in 0.5 ml of RPMI-1640, 10% FCS, Pen/step. The next day, lipids aliquats (1,3 and 5µl of 1mg/ml liposome in water) were diluted in polystyrene tubes containing 100µl of serum-free, antibiotic-free RPMI-1640 and to these tubes were added 150 ng of plasmid in 100µµl of the same medium (suboptimal amount in polypropylene tubes) and incubate for 15 min. The cells were washed twice with Dulbecco's PBS, the lipid:plasmid complexes added to them and then incubated for 7h at 37 C^o in 5% CO_2 atmosphere. Growth medium was added to the cells for a final volume of 1 ml and a final concentration of 10 % FCS, pen/strep, 50ug/ml gentamicin in RPMI-1640 and were incubated overnight.

Example 21: Transfection of Primary Human Tracheobronchial and Epidermal Keratinocytes.

Cells were grown in serum free medium (SFM) and plated on 35 mm plates (6wells) such that the confluence after 24h was above 50%. Plasmid reporter (2μg and 5μg, respectively) was mixed with variable amounts of liposomes (see tables IV and V) and the complex formed added to the cells. The cells were transfected during 4h and 6h, respectively. The DNA/liposome complex was removed by rinsing with SFM and the cells incubated for 48h under normal growth conditions and then assayed for the appropiate marker.

Example 22: Transfection and CAT assay of Jurkat Cells (suspension cells).

The cell suspension culture was transferred to a 50 ml conical tube and centrifuged at 400g for 10 min. The cells were washed twice by aspirating off the supernatant and gently resuspending the cell pellet in 25 ml of sterile PBS and centrifuging again at 400g for 10min. The pellet was resuspended in a volume of serum-free growth medium such that a final concentration of 6.25×10^6 cells /ml is obtained (about 10 ml). 35 mm cell culture plates were inoculated with 0.8 ml of the cell suspension. For each well, 10 μg of CAT plasmid were dissolved in 110 μl of serum-free medium and a separately in another tube were diluted 30 μl of the lipid solution in 70 μl of serum-free medium. The plasmid and lipid solution were mixed and gently swirled and let stand at room temperature for 10 min. The complex DNA/lipid solution was then randomly dropped over the culture well. The wells were gently swirled and then incubated at 37 ^{0}C under a 7 % CO_2 atmosfere for 5 hours. After 5h incubation, 4ml of 12.5 % FBS growth medium were added to the wells and the incubation continued for additional 72 h under the above conditions. The cells were then transferred to 10 ml Falcon tubes and the wells rinsed with 5ml of sterile PBS . The cell suspension was washed twice with 5ml of sterile PBS as previously. The final pellet was resuspended in 400 μl of lysis buffer and transferred to 1.5 ml centrifugation tubes. The tubes were capped and placed horizontally on a rocker and the cells lysated for 30 min.

100 µl aliquats were then assayed for CAT activity following the procedure of Neumann et al. (1987) *Biotechniques* 5: 444

Example 23: Assay for transient transfection (adherent cells).

The cells were washed twice with Dulbecco's PBS and stained with freshly prepared fixative (2%formaldehyde/ 0.2% glutaraldehyde in PBS) for 5 min, washed twice with Dulbecco PBS.). Then, stained with 0.5 ml of β-galactosidase histochemical stain (0.1% x-gal, 5mM potassium ferrocyanide, 5mM potassium ferrocyanide, 2mM MgCl2 in PBS) for 24h at 37 C° in a 5% CO_2 atmosphere. Blue cells (β-gal positive) were counted.

Example 24: Results and Discussion.

Results are summarized on tables I , II ,III, IV and V. Tables I and II show the relative transfection efficiency of compounds 5a, 6d and 13d versus control compound TMTPS (Compound 3 in PCT Int.Pub.No. WO95/17373. Haces, A. et al.) in HepG2 (human hepatocarcinoma) and HeLaS3 (human cervical carcinoma) cells, respectively. And under suboptimal conditions for activity. In these cell lines, compounds 6d and 13d show a 2-2.4 fold higher efficiency than the TMTPS control , and compound 5a is half as active as control in HepG2 cells and showed negligible activity in HeLaS3 cells. Table III shows an analogous comparison using Jurkat cells (T-cell leukemia). In this experiment, compounds 5a and 13d show similar efficiency as TMTPS, but compound 6d shows almost 38% more activity than that of the control. Tables IV and V shows the relative efficiency of compounds 6d and 13d in the primary human tracheobronchial epithelial and human keratinocytes cells. Primary cells are cells that are freshly isolated from humans or animals and which , unlike the cultured cell lines, reflect the potential behavior of a compound in vivo more closely. Thus, for genetic therapy to work, it is necessary to be able to transfect these types of cell lines before any in vivo experiments are tried. These types of cells are also the most difficult to transfect and their transfection efficiencies are usually below 1%. Table IV shows the relative efficiency of compounds 6d and 13d

versus DOTMA (Lipofectin™ Reagent, Life Technologies, Inc.,Supra) in primary human Tracheo brochial cells. Both of these compounds show a relative range of activities of 5.3 to 6.0 times higher than that of the Lipofectin control. At the same time their cell toxicity was below 5%, unlike the control which showed toxicity in the 10-20% range. Thus, these lipid reagents are superior to the commercial standards in both respects. In addition, this is a very significant result since tracheobronchial cells are involved in the genetic disease cystic fibrosis. There are several genetic therapy clinical trials being conducted at the present time targeting these cells using either viral or liposomal vectors (see 10th Annual North America Cystic Fibrosis Conference, Orlando, Fl., Oct 24-27 (1996), Abstracts or *Pediatr Pulmonol* Suppl, 13: 74-365, Sept, 1996) .

Table V depicts the the percentages of β-gal positive cells (absolute number) which were obtained in primary human epidermal keratinocytes with compounds 5a and 6d versus that obtained with DOSPA control (Lipofectamine™ Reagent, Life Technologies, Inc. Supra) . Compounds 5a and 6d gave, respectively, 35% and 50% positive cells as compared with 2% positives for the control. This represents a 15-25 fold better efficiency for these novel liposome reagents when compared with this well known standard. Moreover, primary human keratinocytes are also a potential target cells for genetic therapy (Fenjves, E.S. et al., *Hum Gene Ther* 5: 10,1241-8, Oct. 1994.), but its use has been restricted due to the lack of highly efficient transfection vectors.

TABLE I

TRANSFECTION OF HEP G2 CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount(μg)	β- Gal Positive Cells (%)
Compound 5a (1: 1.8)	3 μg	0.8
Compound 6d (1: 1.5)	3 μg	4.3
Compound 13d (1:1.5)	3 μg	2.9
Control TMTPS/DOPE(1:1.5)	5 μg	1.5

Cells were plated in 48 well plates at a density of 1 x 10^5 per well in 0.5 ml of growth medium. After 24h, the cells were washed with serum free medium and transfected with a **suboptimal** amount (150 ng) of plasmid pCMV-βgal. using 1,3 and 5ul (1,3 and 5μg) of lipid formulation. The amount giving the highest level of transfection efficiency is shown. The experiment was run in triplicate.

TABLE II
TRANSFECTION OF He La S3 CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	β-Gal Positive Cells (%)
Compound 5a (1:1.8)	5 μg	0.001
Compound 6d (1:1.5)	3 μg	2.4
Compound 13d (1:1.5)	1 μg	2.1
Control TMTPS/DOPE(1:1.5)	5 μg	0.9

Cells were plated in 48 well plates at a density of 1 x 10^5 per well in 0.5 ml of growth medium. After 24h, the cells were washed with serum free medium and transfected with a **suboptimal** amount (150 ng) of plasmid pCMV-βgal. using 1,3 and 5ul (1,3 and 5μg) of lipid formulation. The amount giving the highest level of transfection efficiency is shown. The experiment was run in triplicate.

TABLE III
TRANSFECTION OF JURKAT CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	CAT Activity (mU/well)
Compound 5a (1:1.8)	30 μg	196.00
Compound 6d (1:1.5)	30 μg	298.20
Compound 13d(1:1.5)	30 μg	220.00
Control TMTPS/DOPE(1:1.5)	30 μg	216.44

Wells were inoculated with 6.25 x 10^6 cells . 10 μg of CAT plasmid were mixed with 30μg (optimal amount known for the control) of the lipids and then added to the cells. After 5h the transfection was quenched with FBS containing medium and the cells incubated for 72h. Cells were lysated in 400 μl of buffer. 100 μl aliquats were assayed for CAT activity.

TABLE IV
TRANSFECTION OF PRIMARY HUMAN TRACHEOBRONCHIAL EPITHELIAL CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	Luciferase Activity (counts)
Compound 6d (1:1.5)	12 μg	7297022
Compound 13d (1:1.5)	12 μg	8343975
Control , Lipofectin™*	6 μg	1379341

35 mm plates were inoculated with cell isolates and transfected at 90% confluence. 2μg of firefly luciferase plasmid were mixed with 12 μg of the lipids and then added to the cells. After 5h, the transfection was quenched by removal of the DNA/Lipid complex and the cells incubated for 72h. Cells were lysated and aliquats assayed for luciferase activity. Cell toxicity , determined by the trypan blue method, was below 5% for lipids 6d and 13d and between 10-20% for Lipofectin.* Lipofectamine was also run as a control, but its efficiency was negligible.

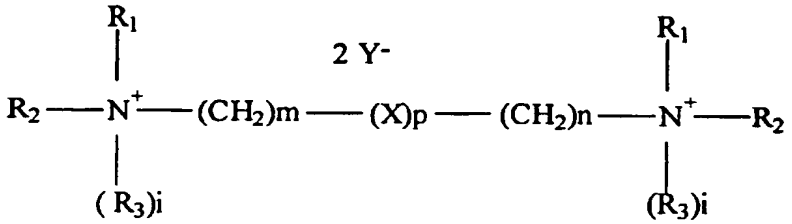
TABLE V
TRANSFECTION OF PRIMARY HUMAN EPIDERMAL KERATINOCYTES

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	β-Gal Positive Cells (%)
Compound 5a (1:1.5)	40 μg	35 %
Compound 6d (1:1.5)	20 μg	50 %
Control , Lipofectamine™	25 μg	2 %

Cells were seeded at $2x10^5$ /well in 35 mm wells and transfected the next day. 5μg of βgal DNA were mixed with the appropiate amount of lipids and added to the cells. After 4h, the medium was replaced and the cells incubated for additional 48h and then assayed. Blue cells were observed under the microscope and counted.

What is Claimed:

1. A composition comprising the mixture of a compound having the structure:



Y= Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion

X = O,S(O),CH_2 R_1,R_1' = independently: C_1-C_{18} linear hydrocarbon.

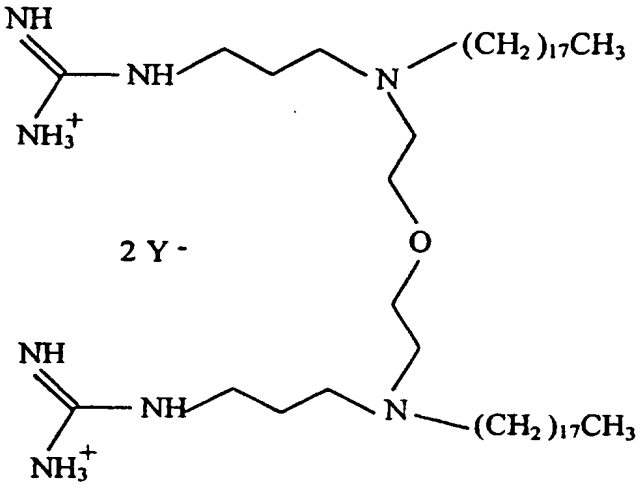
m,n = 1,2,3 R_2,R_2' = independently : H ; C_1-C_{18} linear alkyl; cyanoethyl; aminopropyl; aminobutyl; C_2 -C_4 alkyl guanidinium or amidinium; N,N,N- independently (C_1-C_{18})aminopropyl or aminobutyl; C or N substituted spermine or spermidine; N,N-(C_4-C_{18})alkyl-4- aminonobutyrylaminopropyl

p = 0, 1 R_3,R_3' = independently: C_1-C_6 linear alkyl, acetoxyethyl,
i = 0, 1 $CH_2CO_2CH_2CH_3$

$X \neq CH_2$ when R_2,R_2' = C_1-C_6 linear alkyl
When p = 1, m and $n \neq 1$

and a member of the class consisting of nucleic acids, oligonucleotides, mononucleotides, polypeptides and proteins.

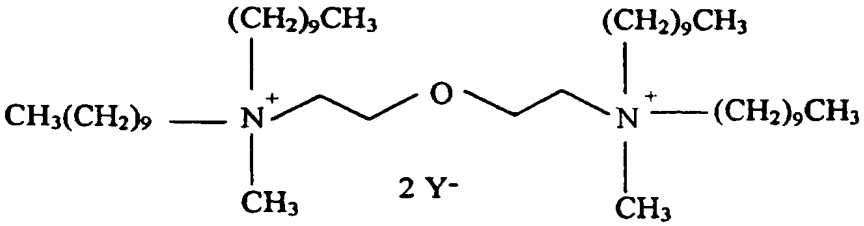
2. A composition comprising the mixture of a compound having the structure:



Y = Cl^-, Br^-,I^-, AcO^-, or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

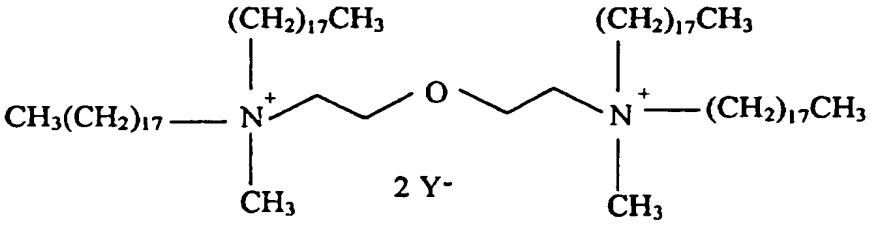
3. A composition comprising the mixture of a compound having the structure:



Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

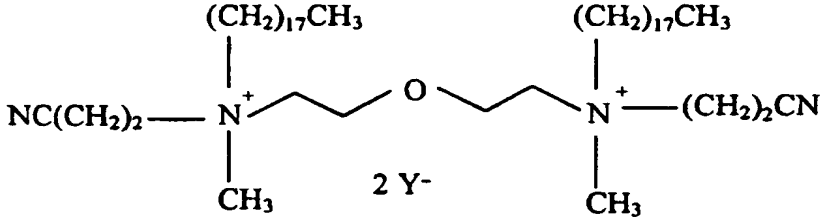
4. A composition comprising the mixture of a compound having the structure:



Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

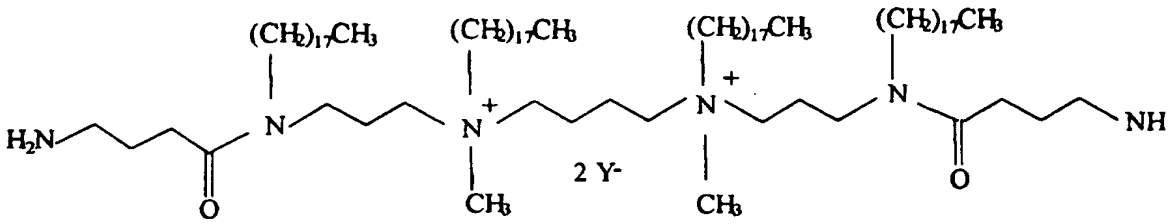
5. A composition comprising the mixture of a compound having the structure:



Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

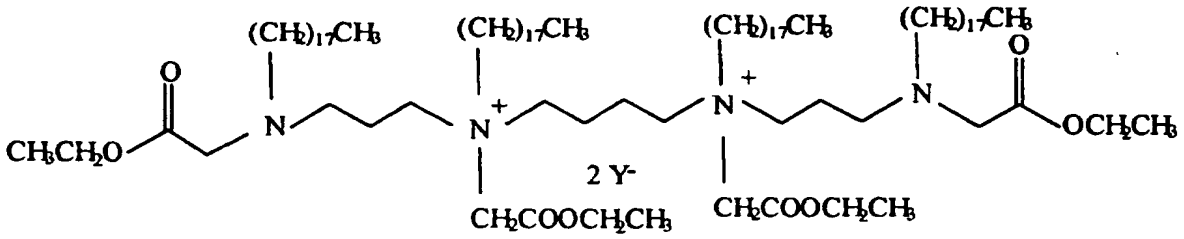
6. A composition comprising the mixture of a compound having the structure:



Y = Cl, Br,I, AcO, or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

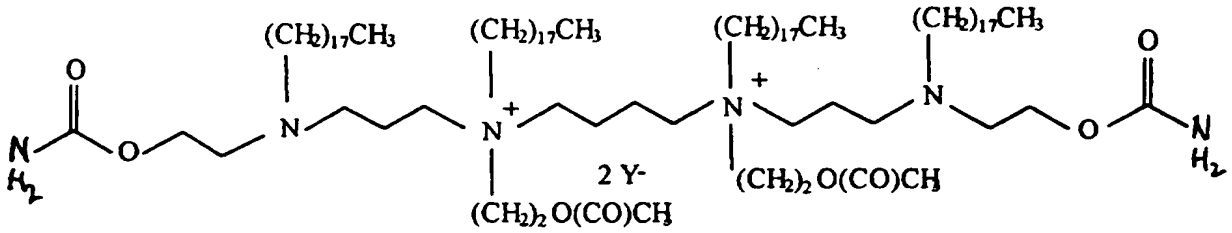
7. A composition comprising the mixture of a compound having the structure:



Y = Cl, Br,I, AcO, or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids, oligonucleotides,mononucleotides, polypeptides and proteins.

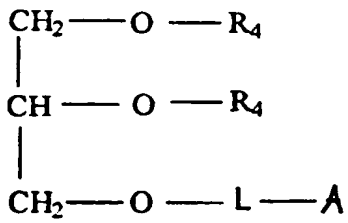
8. A composition comprising the mixture of a compound having the structure:



Y = Cl-, Br-, I-, AcO-, or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

9. A composition comprising the mixture of a compound having the structure:



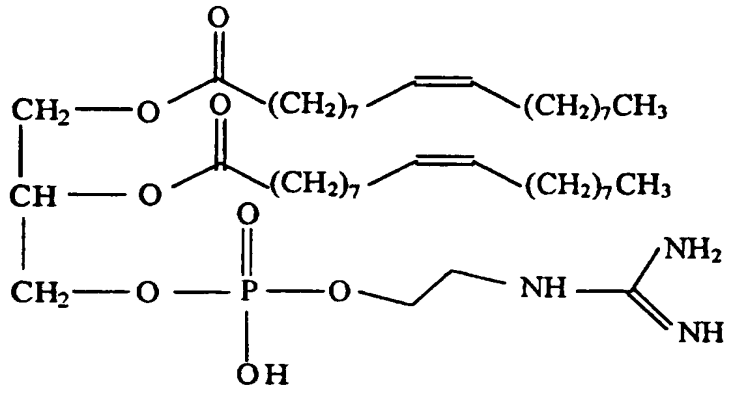
R_4 = Independently: linear $(CO)C_6$-C_{20}, C_6-C_{20}

L = 2-10 atoms linker

A = Guanidinium; imidinium; guanidylated polyamines.

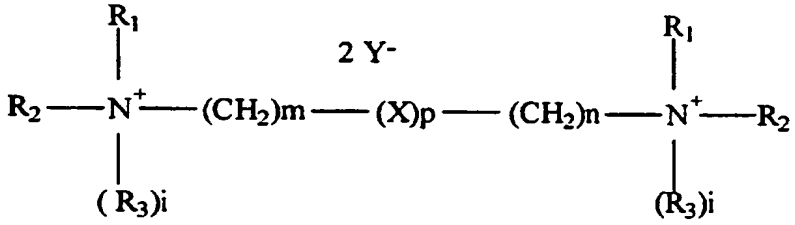
and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

10. A composition comprising the mixture of a compound having the structure:



and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

11. A compound having the formula:



Y= Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion

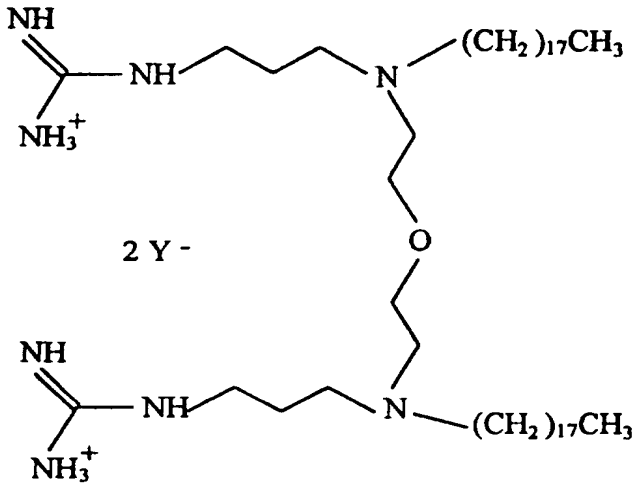
X = O,S(O),CH_2 R_1,R_1' = independently: C_1-C_{18} linear hydrocarbon.

m,n = 1,2,3 R_2,R_2' = independently : H ; C_1-C_{18} linear alkyl; cyanoethyl; aminopropyl; aminobutyl; C_2 -C_4 alkyl guanidinium or amidinium; N,N,N- independently (C_1-C_{18})aminopropyl or aminobutyl; C or N substituted spermine or spermidine; N,N-(C_4-C_{18})alkyl-4- aminonobutyrylaminopropyl

p = 0, 1
i = 0, 1 R_3,R_3' = independently: C_1-C_6 linear alkyl, acetoxyethyl, $CH_2CO_2CH_2CH_3$

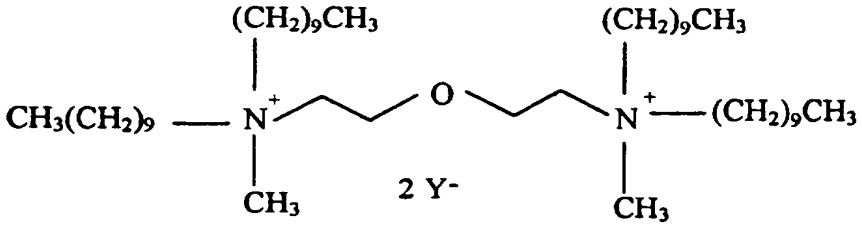
$X \neq CH_2$ when R_2,R_2' = C_1-C_6 linear alkyl
When p = 1, m and $n \neq 1$

12. A compound having the structure:



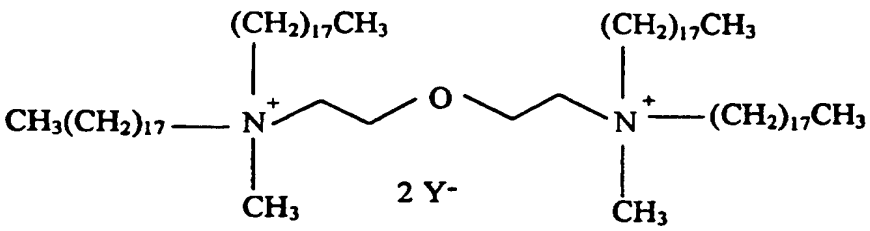
Y = Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion.

13. A compound having the structure:



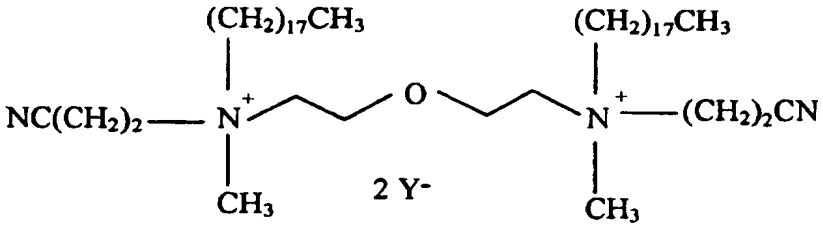
Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion.

14. A compound having the structure:



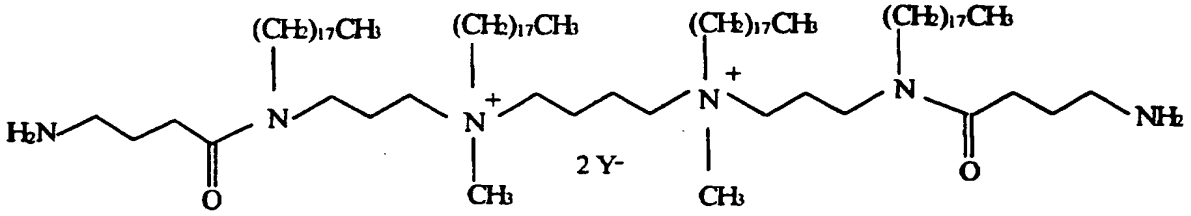
Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion.

15. A compound having the structure:



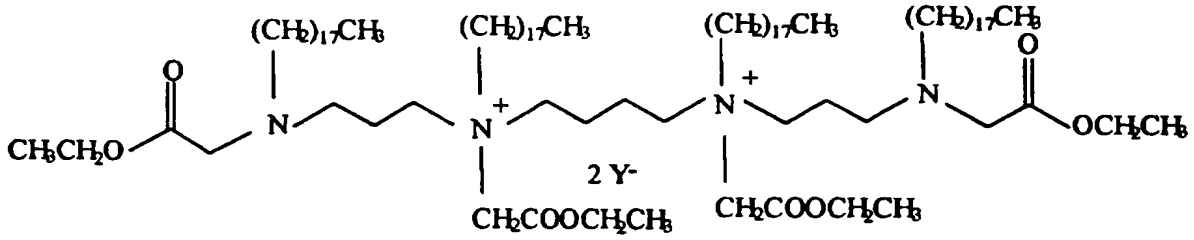
Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion

16. A compound having the formula:



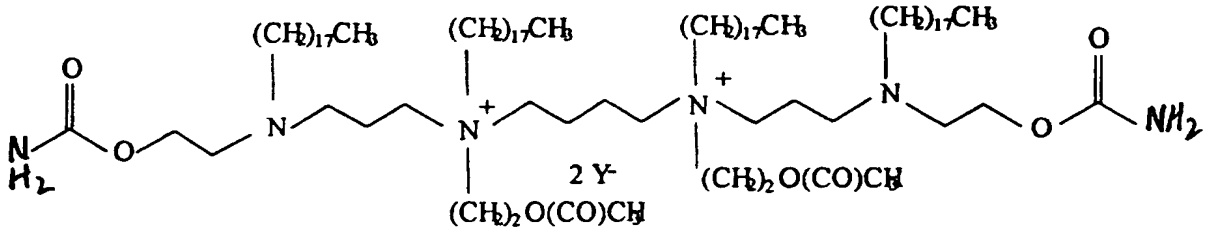
Y = Cl^-, Br^-,I^-, AcO^-, or any pharmaceutically acceptable anion.

17. A compound having the structure:



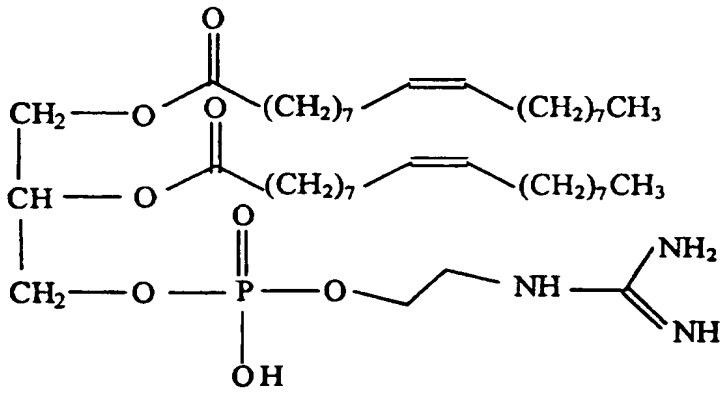
Y = Cl^-, Br^-,I^-, AcO^-, or any pharmaceutically acceptable anion.

18. A compound having the structure:

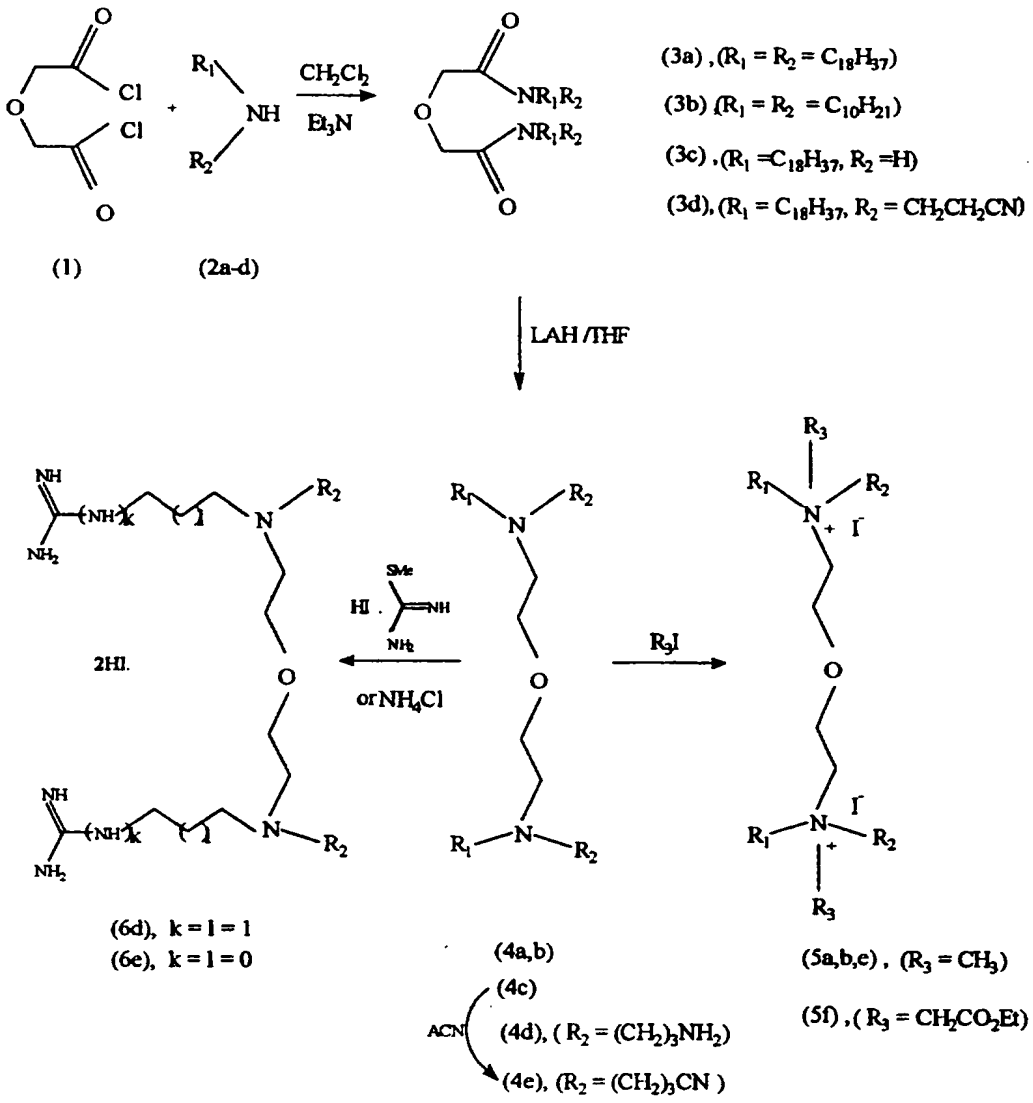


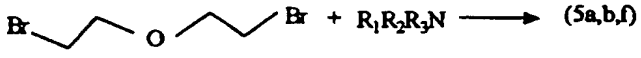
Y = Cl, Br,I-, AcO, or any pharmaceutically acceptable anion.

19. A compound having the structure:



SCHEME I





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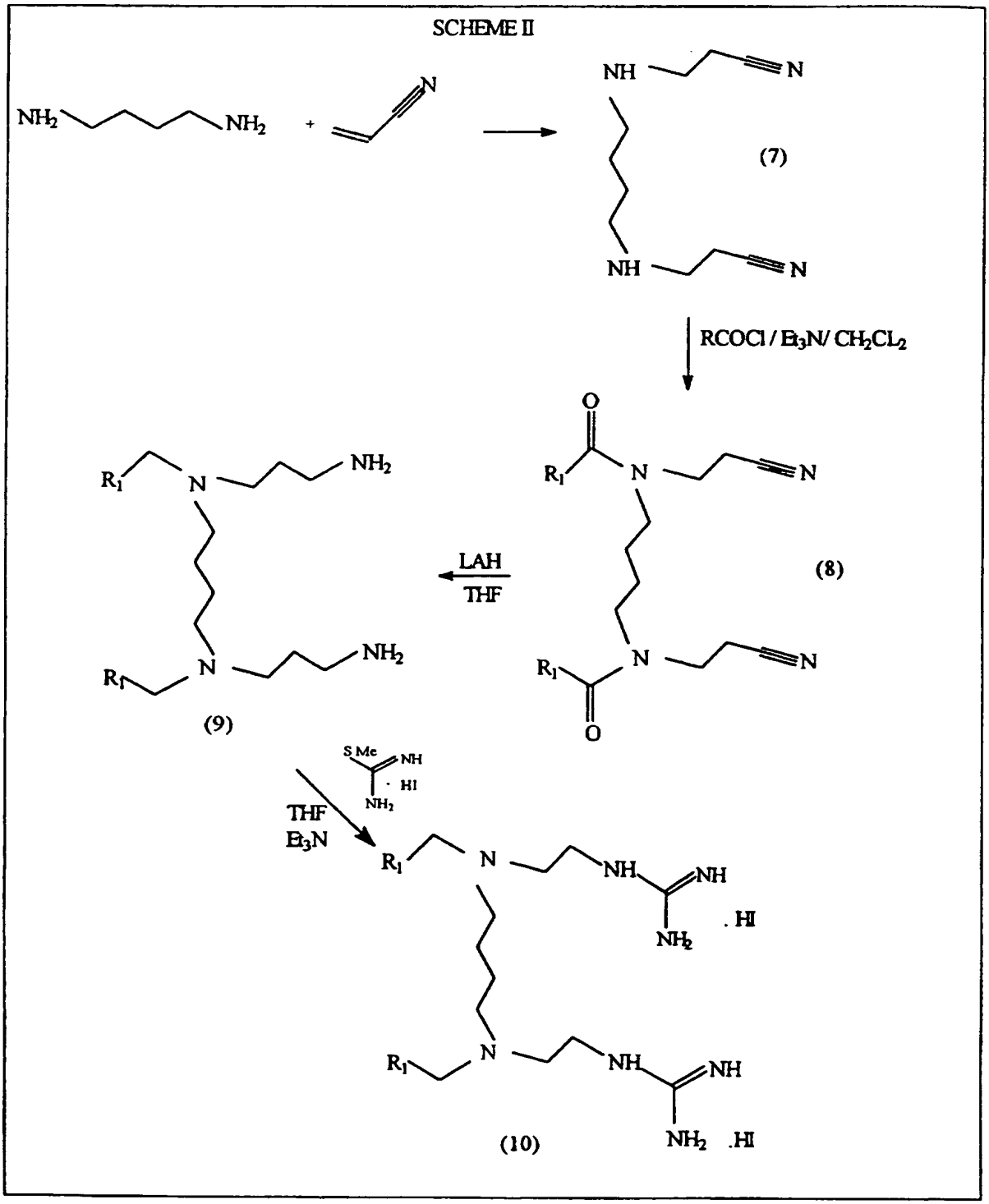

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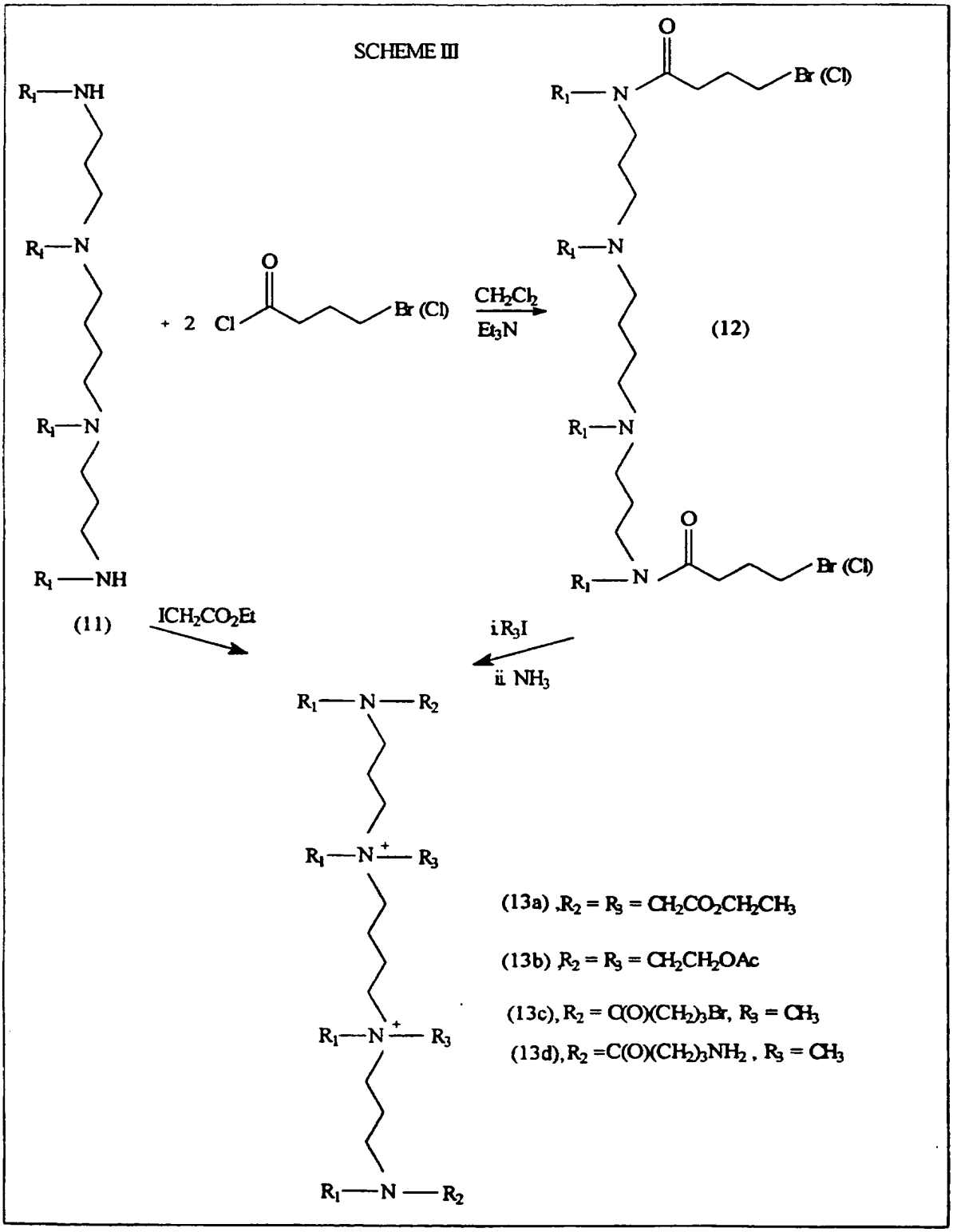

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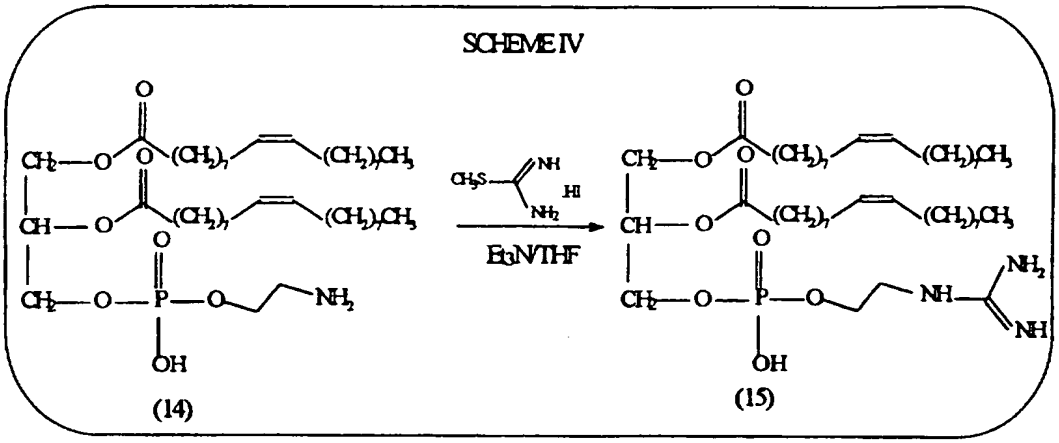

(14) (15)

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A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 37/18; A61K 37/16
US CL :514/2,7

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2,7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG: CAS, Medline, Derwent Biotechnology Abstracts, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,459,127 A (FLEGNER et al.) 17 October 1995, see entire document.	1-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Published
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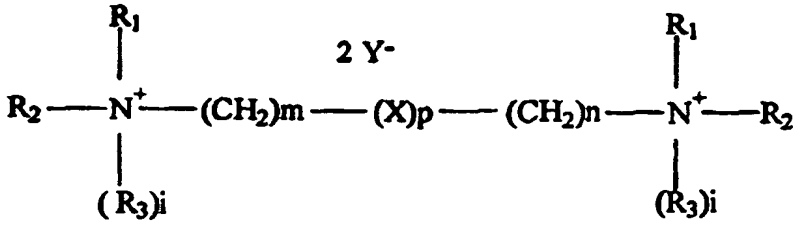
(54) Title: CATIONIC LIPIDS FOR TRANSFECTION OF NEGATIVELY CHARGED OR NEUTRAL MOLECULES INTO LIVING CELLS

(57) Abstract

A cationic lipid for transfection of nucleic acids comprising the mixture of a nucleic acid with a compound having structure (I), wherein Y = Cl-, Br-, I-, AcO-, or any pharmaceutically acceptable anion; X = O, S(O), CH_2; R_1, $R_{1'}$ = independently: C_1-C_{18} linear hydrocarbon; m, n = 1, 2, 3; R_2, $R_{2'}$ = independently: H; C_1-C_{18} linear alkyl; cyanoethyl; aminopropyl; aminobutyl; C_2-C_4 alkyl guanidinium or amidinium; N,N,N- independently (C_1-C_{18})aminopropyl or aminobutyl; C or N substituted spermine or spermidine; N,N-(C_4-C_{18})alkyl-4-aminonobutyrylaminopropyl; p = 0, 1; i = 0, 1; R_3, $R_{3'}$ = independently: C_1-C_6 linear alkyl, acetoxyethyl, $CH_2CO_2CH_2CH_3$; X ≠ CH_2 when R_2, $R_{2'}$ = C_1-C_6 linear alkyl; when p = 1, m and n ≠ 1.



(I)

* (Referred to in PCT Gazette No. 10/1998, Section II)

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CATIONIC LIPIDS FOR TRANSFECTION OF NEGATIVELY CHARGED OR NEUTRAL MOLECULES INTO LIVING CELLS

Field of the Invention

This invention relates to polycationic lipids useful for the delivery (transfection) of nucleic acids (DNA,RNA) and other negatively charged or neutral molecules into living cells, either *in vivo* or *in vitro*.

Background of the Invention

Liposomes aggregates made with polycationic lipids are useful structures capable of complexing with negatively charged macromolecules such as DNA or RNA. These complexes can be taken up by living cells and then , once inside the cytosol, through an unknown mechanism, they are presumed to migrate into the cell nucleus. In the nucleus, there are enzymes capable of "reading" and "expressing" the message coded by the nucleic acids so delivered and produce new proteins, which were not being produced by the cell before the transfection of the foreign nucleic acid. When cells so transfected divide and their daughter cells still have the capability to produce the proteins encoded by the initially transfected DNA , the transfection is said to be stable. That is, the new DNA has stably integrated into the cell nucleus changing the cell's genetic make-up. If , on the other hand, the parent cells can produce the protein encoded by the transfected DNA, but their daughter cells are not capable of expressing the such DNA , the transfection is said to be transient. RNA transfection is always transient. Stable transfection of human or animal cells is the basis of the so called gene therapy, since cells which are deficient on a crucial protein for the organism's survival could be in principle repaired by stably transfecting the DNA needed to produce the absent protein. Another type of potential use of DNA/RNA transfection for therapy is the antisense therapy . In this approach, a short piece of nucleic acid (oligonucleotide) capable of adhering (hybridizing) to defective DNA (or RNA) which is being expressed by the cells to produce an undesired protein, such as an oncoprotein (cancer causing protein), is transfected into the cells in order to stop the expression of the undesired protein by virtue of its adherence to the defective nucleic acid. This method of therapy does not change the genetic make-up of the cell, but blocks the effect of the genetic disorder already present in the cell's genome. Besides this potential applications of polycationic lipids for use in human therapy, there is already a

well established market for these types of chemicals in the research products field. They are currently being used by researchers to deliver nucleic acids and proteins into cells in order to study how the expression of different genes affect cell growth and function.

There are two possible ways to deliver DNA into cells for gene therapy : *ex vivo* and *in vivo* transfection. In the *ex vivo* modality , cells from a patient are removed from the body, cultured and transfected *in vitro*. Then, the cells are returned into the patient where the beneficial DNA message is hopefully expressed. In the *in vivo* mode, the DNA is delivered directly into the patient, which makes this procedure simpler and less expensive. To date the only effective way to deliver DNA *in vivo* is by using a virus which naturally infects cells of an specific organ (targets that organ) within the body, and whose genetic make up has been modified by adding the DNA beneficial to the patient. Once inside the cells of the patient, the virus can incorporate the new DNA in the genome of the cell (stable transfection) and the parent cell and its daughters can express the beneficial protein. The pathological component of the virus has been deleted before the patient is exposed to such a virus and only the targeting component left intact. Virus can do this process sometimes with nearly 100% efficiency. However, there are risks associated with their use, they can produce immunological reactions which may be fatal to the patient; the DNA incorporation in the cell's genome is random, therefore it might disrupt needed genes or activate oncogenes; they are also difficult to mass produce, etc.

Liposomes or lipid aggregates do not have the side effects of viruses , but are not as efficient as viruses are. There is a constant need to develop newer lipids that can approach the efficiency of viruses without their undesirable side effects (E. Marshall, *Science* **269**,1050 (1995)) . There are several lipids for nucleic acids transfection already in the market. The most relevant of these lipids are: DOTMA (N-[1-(2,3-dioleoyloxy)propyl]- N,N,N-trimethylamonium chloride, U.S. Pat. No. 4,897,355 to D. Eppstein et al.), DMRIE (D,L-1,2-O-dimyristyl-3-dimethylaminopropyl-b-hydroxyethylammoniumchloride, U.S. pat. No 5,264,618 to Felgner, P.L. et al.), DOTAP (1,2-bis(oleoyloxy)-3-3(trimethylammonia)propane) Boehringer-Mannheim Catalog No.1 202 375) , DOGS (5-carboxysperminyglycine dioctadecylamide, U.S. pat. No 5,171,678 to Behr, J-P. et al. DOGS is sold under the trade name Transfectam™ by the Promega

Corp. Madison, WI), DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxyamido)ethyl]-N,N-dimethyl-propanaminium trifluoroacetate, U.S. Pat. No. 5,334,761 to Gebeyehu, G. et al.), DDAB (Dimethyloctadecylammoniumbromide, U.S. Pat. No. 5,279,833 to Rose, J.K.), TMTPS (N,N,N,N-Tetramethyltetrapalmylspermine, PCT Int.Pub.No. WO 95/17373. Haces, A. et al.). DOTMA, DOSPA, DDAB and TMTPS are sold by Life Technologies, Inc., Gaithersburg, MD under the trade names of Lipofectin, LipofectAMINE, LipofectACE and CellFECTIN, respectively. A recent relevant publication which deals with art related to the present invention has been reported by Ruysschaert et al.((1994)*Biochem. Biophys. Res. Commun.*203,1622-1228). All these lipids, except DOGS, are formulated with dioleolylphosphatidylethanolamine (DOPE), which is a neutral lipid devoid of transfection activity, in order to make the active liposomes. These lipids posses some desirable characteristics, however they are far from the ideal vehicle to deliver DNA. Their main drawbacks are low efficiency, non-specificity of targeting, considerably toxicity, low water solubility, and serum inhibition of their action.

Although progress has been made in overcoming some of these obstacles, there is considerable room for improvement an experimentation. The design of these lipids is still a semi-empirical endeavor , since very little is known about the mechanism by which they act.

Therefore, it is the object of this invention to improve the desired characteristics of these lipids by exploring and incorporating new chemical functionalities as well as spatial or topological arrangements which improve the transfection efficiency and lower the toxicity.

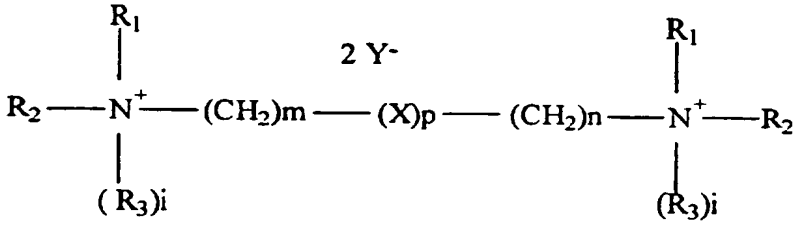
It is also an object of this invention to synthesize polycationic lipids which incorporate a small , non lipid-bilayer-disturbing moiety that mimics a natural molecule, which cells can recognize as their natural effector or ligand, thus facilitating the transfection as well as the specificity of targeting of the macromolecule.

Summary of the Invention

In this invention a series of new polycationic lipids and their method of preparation is described Such lipids are useful as transfection reagents for: nucleic acids, oligonucleotides,mononucleotides, polypeptides and proteins. In addition, some of these lipids are also useful as more effective detergents for cleaning and as vehicles in the cosmetic field.

The present invention describes novel oxo and sulfinyl backbone substituted polycationic lipids with ammonium, guanidinium and imidinium positively charges moieties as anchoring groups having the formula:

Formula I



Y= Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion

X = O,S(O),CH_2 R_1,R_1' = independently: C_1-C_{18} linear hydrocarbon.

m,n = 1,2,3 R_2,R_2' = independently : H ; C_1-C_{18} linear alkyl ; cyanoethyl; aminopropyl ; aminobutyl; C_2 -C_4 alkyl guanidinium or amidinium;N,N,N independently(C_1C_{18})aminopropyl or butyl; C or N substituted spermine or spermidine; N,N-(C_4-C_{18})alkyl-4- aminonobutyrylaminopropyl

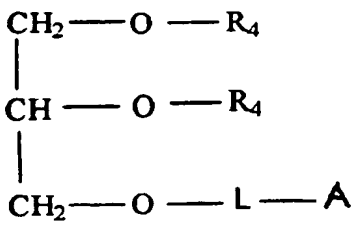
p = 0, 1 R_3,R_3' = independently: C_1-C_6 linear alkyl, acetoxyethyl,
i = 0, 1 $CH_2CO_2CH_2CH_3$

$X \neq CH_2$ when R_2,R_2' = C_1-C_6 linear alkyl

When p = 1, m and n ≠ 1

It is also disclosed in this invention a series of novel phosphatidyl and glyceryl guanidinium cationic lipids having the formula II.

Formula II



R_4 = Independently: linear $(CO)C_6$-C_{20}, C_6-C_{20}

L = 2-10 atoms linker

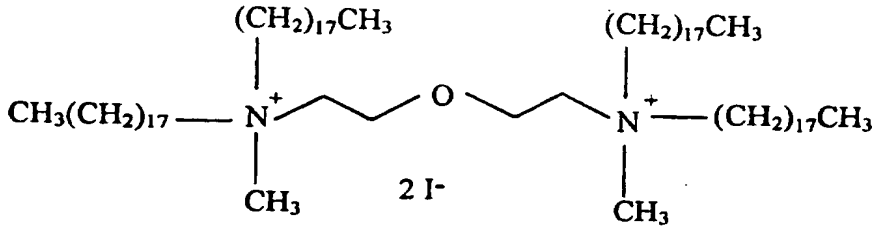
A = Guanidinium; imidinium; guanidylated polyamines.

These compounds can be used alone or in mixtures with other liposome forming compounds (co-lipids) to prepare lipid aggregates which are useful to deliver macromolecules, specifically negatively charged macromolecules to living cells either in culture or *in vivo*.

Compounds of Formula I:

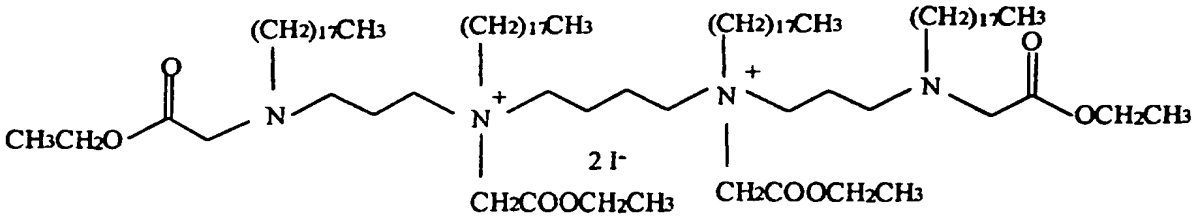
The lipids depicted in Formula I have a hydrocarbon backbone substituted with heteroatoms which are sterically smaller, but equally or more flexible as the methylene group that they replace. This feature makes these new lipids fit more closely to the macromolecule to be delivered to the cells. This closer fit combined with the polycationic nature of the backbone produces a tighter binding. In addition, these heteroatoms are hydrophilic; thus, they not only confer an increased amphiphilic character to the lipids but also make the backbone more linear or "stretched" as compared to the all-methylene groups backbone. The latter being hydrophobic tends to wrap around itself in an aqueous environment, therefore pulling the positively charged moieties away from the negatively charged phosphates on the DNA/RNA backbone, this results on a weaker binding between the polycationic lipid backbone and the polyanionic DNA backbone, since the opposite charges can not align properly in this arrangement. The hydrophilic backbone being linear allows for proper alignment of the opposite charges, also leading to a tighter

binding. In addition, the higher hydrophilicity conferred by the heteroatoms on the polycationic backbone, make possible the addition of more hydrophobic tails without loss of water solubility, thus making these compounds more densely packed than compounds of the prior art. This is an advantage since the same molar amount of lipid will produce a higher hydrophobic coating of the nucleic acid to be delivered. In fact, one of the preferred embodiments is compound (5a) which has four hydrophobic tails and two positive charges (two tails per charge).

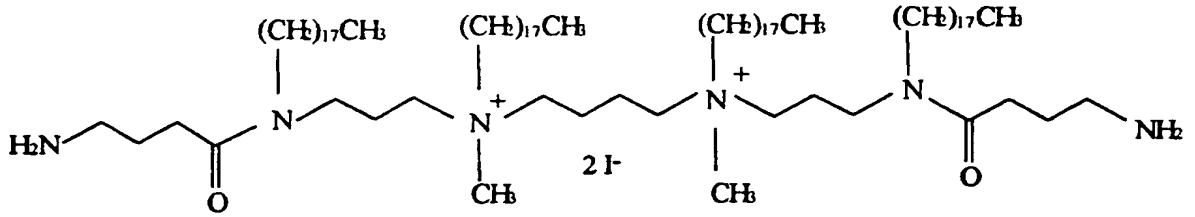


(5a)

Some of the compounds herein described, such as (13a,b) have fewer hydrophobic tails and a more hydrophobic backbone (no heteroatom substitution, but less hydrophobic overall). However, these compounds have moieties which mimic small natural biological effectors such as the neurotransmitters gamma amino butyric acid (GABA), acetylcholine etc. These moieties bind to their corresponding cell receptors targeting the delivery of nucleic acids to those cells rich in these type of receptors such as muscle and neural cells. The latter type of cells are among the most difficult to transfect since they are postmitotic cells (non-dividing). These small chemical moieties do not perturb the ability of the lipids to form liposomes aggregates and at the same time confer more amphiphilic character to said lipids, since they are polar entities. A particular preferred embodiment of the latter compounds are compounds of formulae (13a) and more specifically compound (13d).



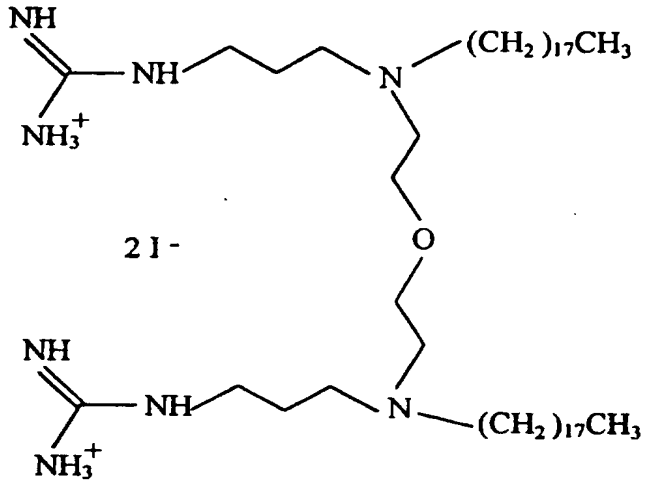
(13a)



(13d)

Another novel feature of the compounds disclosed here is the fact that, in addition to the traditional quaternary ammonium salts, guanidino and amidino moieties are used as permanent positively charged centers. These functional groups are strongly basic an have the same charge as their ammonium counterparts, but have the advantage of being sterically smaller , since they are planar. Thus, they can get closer to the negatively charged phosphates of the DNA/ RNA backbone producing a stronger binding interaction than that of ammonium salts. Furthermore, these guanidinium and amidinium moieties have the ability to form hydrogen bridges with the nucleic acids bases (guanidinium salts are used as chaotropic agents to precipitate DNA) therefore they have an additional binding mode not available to ammonium salts. Moreover, the guanidino moiety can also be used to target neural cells, since compounds such as Guanethidine , which possess such a functional group, are internalized by neurons (Wiener, N. In, *The Pharmacological Basis of Therapeutics*, (Gilman, A.G.; Goodman,L.S.; Rall, T.W.; and Murad, F.; Eds.) Macmillan Pubs. Co. New York, 1985,pp. 181-214.) . Thus , by including the lipidic content as well as the amine and guanidino moieties of Guanethidine in our novel

liposome reagents we can target this difficult to transfect cell type. A particularly useful and preferred embodiment of these compounds is compound (6d), which is the most active of compounds tested.

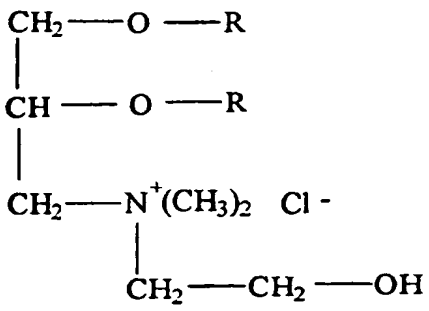


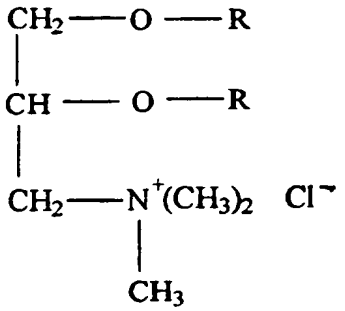
(6d)

Additionally, reduced or no toxicity was observed for these lipids at the concentrations tested.

Compounds of Formula II:

Despite all the reasons given above in order to "rationally design" these lipids, it is still impossible to predict their DNA transfection activity at this time. In fact, cationic lipids for DNA transfection already in the market such DOTAP, DOTMA, DMRIE and DORI whose chemical structures are almost identical to that of the cationic lipid known as the Rosenthal Inhibitor. (Rosenthal, A.F. and Geyer, R.P., *J. Biol. Chem.* 235(8):2202 (1960)) have significant transfection activity, unlike the Rosenthal Inhibitor which is reported to be inactive as a DNA transfection reagent (see U.S. pat No 5,264,618 to Felgner, P.L. et al.). The following formulae illustrate this point more clearly :



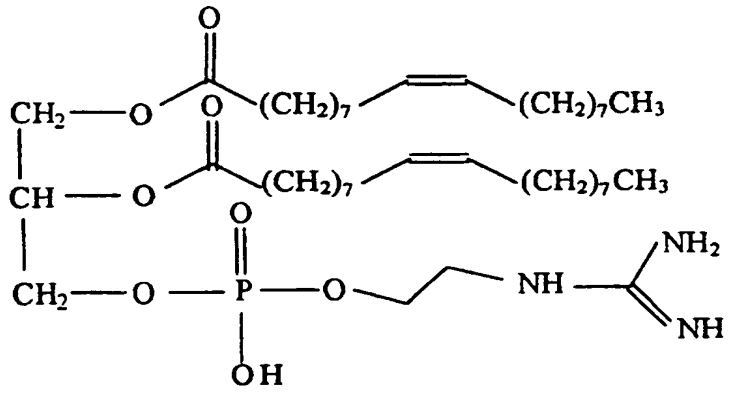


R = Stearoyl, Rosenthal inhibitor (inactive)
R = Myristyl, DMRIE (active)
R = Oleoyl, DORI diester (active)

R = Oleyl, DOTMA (active)
R = Oleoyl, DOTAP (active)

Compounds of formula II described herein have also a similar structure to that of the Rosenthal Inhibitor. However, these compounds differ from the Rosenthal Inhibitor on that a guanidinium or amidinium functionality is used as the positively charged anchoring group, and they also lack a quaternary ammonium group at the C-1 position of the glycerol backbone. A preferred embodiment of these latter type of transfection reagents is compound (15).

(15)



An interesting feature of this compound is that it has the ability to form liposomes without the need of co-adjuvants such as DOPE or DOPC. Thus, it can be used to form liposomes with other cationic lipid compounds.

Specific Examples of the Invention.

Scheme I

This reactions scheme shows the general synthetic route to prepare polycationic lipids having a heteroatom substituted anchoring backbone. Thus, diglycolyl chloride (1) is treated with a suitable primary or secondary amine (2a-d) in methylene chloride in the presence of a base such as triethyl amine under an inert gas such as argon at room temperature to obtain the corresponding diglycolamides (3a-d). These amides can then be reduced with lithium aluminum hydride or borane in refluxing anhydrous tetrahydrofurane (THF) to afford the corresponding amines (4a-d). Secondary amine (4c) was easily converted to the corresponding tertiary amine (4e) upon treatment with acrylonitrile. Compound (4e) can be treated with ammonium chloride at high temperature to produce the corresponding amidine(6e). Alternatively, the latter amidine derivatives can be obtained by reacting the dinitrile (4e) with anhydrous hydrogen chloride in ethanol, followed of treatment of the imidoester so obtained with ammonium hydroxide. Primary amine (4d) can be converted to the target compound (6) by treatment with S-methyl isothiouronium hydroiodide (S-methyl thiourea) in tetrahydrofurane in the presence of triethylamine. Additionally , this guanidinium derivative can be alkylated with for example iodo methane to produce the corresponding quaternary ammonium salt. Tertiary amines (4a,b,e) are treated with an alkylating agent such as iodo methane , iodoethyl acetate or 2- bromo ethyl acetate to afford the quaternary ammonium salts (5a,b,f). The latter compounds were also synthesized by treating the corresponding tertiary amines with the commercially available 2-bromoethyl ether (lower panel, scheme I). This route has only two steps , but is not as flexible or prolific as the route depicted on scheme I.

Scheme II

Compound (7) is easily synthesized by treating commercially available 1,4-diaminobutane with acrylonitrile. Diamide-dinitrile (8) is then easily obtained by treatment of compound (7) with an acyl halide such as palmitoyl chloride in methylene chloride in the presence of triethyl amine. The diamide-dinitrile (8) can be reduced with lithium aluminum hydride or borane in THF to the corresponding tertiary and primary amines

functionalities to afford compound (9). Guanidinium compound (10) can be obtained in a similar fashion as shown in scheme I for compound (6d) by reacting the primary amines of compound (9) with S-methyl thiourea in THF and triethylamine.

Scheme III

Tetrapalmyl spermine (11) (Haces, A. et al. PCT Int. Pub. No WO 95/17373) was treated with ethyl iodoacetate at room temperature to afford the tetraalkylated derivative (13a). Similarly, compound (11) can be treated with 2-bromoethyl acetate at high temperature to afford (13b). Reaction of (11) with 4-bromo or 4- chloro butyryl chloride in methylene chloride in the presence of triethyl amine at low temperature gives the corresponding 4-bromobutyramide derivative (12). Intermediate (12) is immediately treated with iodo methane to produce the N,'N"- dimethylated intermediate (13c) which in turn is treated with an excess of ammonium hydroxide in tetrahydrofurane at elevated temperature to convert the bromide (or chloride) into the corresponding primary amine (13d). All these compounds have moieties which resemble or mimic the neurotransmitters gamma aminobutyric acid (GABA, compound (13d)) and acetyl choline (compounds (13a,b)). These small groups do not change substantially the liposomal forming ability of the lipid molecule and at the same time are capable of being recognized by neural or muscles cells. This preferential recognition by these type of cells makes these lipids target specific DNA/RNA delivery agents.

Scheme IV

Commercially available dioleoylphosphatidylethanolamine (14) was treated with an excess of S-methyl isothiouronium hydroiodide in tetrahydrofurane and in the presence of triethylamine to afford the corresponding guanidinium compound (15).

EXAMPLES

Example 1: Synthesis of octadecylcyanoethylamine (2d).

Octadecylamine (2g, 7.4 mmol) and acrylonitrile (15ml) were heated for 3h at 70°C in a thick wall test tube capped with a teflon lined cap. TLC (silica gel; ethyl acetate) shows a new spot at rf = 0.65 . The excess acrylonitrile was removed in vacuo to afford pure product (2.40g, 100% yield). H-NMR ($CDCl_3$) δ: 0.88 (t, 3H), 2.25 (br.s.,32H), 2.53 (t, 2H), 2.62 (t,2H), 2.93 (t,2H). FT-IR (cm^{-1}) 2250 (CN).

Example 2: Synthesis of bis (mono and dialkyl)diglycolamides(3a-d), general procedure.

To a solution of dialkylamine (2mmol) and triethylamine (2 mmol) in methylene chloride (250ml) under argon was added diglycolyl chloride (1mmol) and the resulting solution was stirred for 18h at room temperature. TLC (silica gel; MeOH or CH_2CL_2/THF,3:1) shows absence of starting material and a new spot. The methylene chloride solution was washed with sodium bicarbonate (10% in water), dried (Na_2SO_4) and the solvent removed, to afford the desired diamide.

Proceeding as described before and using the appropriate mono or dialkylmine the following compounds were prepared:

3a. Bis (dioctadecyl) diglycolamide (81% yield), H-NMR (CDCL3) δ: 0.88 (t,12H), 1.25 (s, 124H), 1.5 (br.s, 8H), 3.15(t,4H), 2.9 (t,4H), 3.2 (s, 4H). R (cm^{-1}): 1651 (C=O);

3b. Bis (didecyl)diglycolamide (73% yield), H-NMR(CDCL3)d X: 0.85 (t,12H), 1.25 (s, 64H), 1.5 (br.s,8H), 3.18 (t,4H), 3.3 (t,4H), 4.4 (s, 4H). FT-IR(cm^{-1}): 1657(C=O);

3c. Bis (octadecyl)diglycolamide (100% yield),H- NMR(CDCL3) δ: 0.85 (t, 6H), 1.25 (br.s, 60H), 1.5 (br.s,4H), 3.3 (q,4H), 4.05(s,4H), 6.4 (br.s,2H);

3d. Bis (octadecylcyanoethyl) diglycolamide (98%yield), H-NMR ($CDCl_3$) δ: 0.85 (br.t, 6H), 1.1-1.6 (br.s, 64H), 2.65 (t, 4H), 3.25 (br.t, 4H), 3.55 (br.t, 4H), 4.3 (s, 4H);

Example 3: Synthesis of N,N,N',N'_-Mono and dialkyl 2,2'-oxybis ethylamines (4a-d), general procedure

To a solution of lithium aluminum hydride (6 to 64 molar excess) in dry tetrahydrofurane (THF) was added the corresponding diamide in small portions under a blanket of argon. The resulting mixture was refluxed for two to three days under argon. The progress of the reaction was followed by TLC (silica gel; CH_2Cl_2/TFH, 3:1 for dialkylamides; 10% triethylamine in CH_2Cl_2 for monoalkylamides and triethylamine for cyanoethylamides). The reactions were quenched with sodium hydroxide (10% in water). The mixture was filtered, the filtrate dried (Na_2SO_4), and the solvent removed in vacuo to afford the desired products.

Proceeding as described above and using the appropriate diamide the following compounds were obtained:

4a. N,N,N',N'-dioctadecyl-2,2'-oxobis ethylamine (77% yield), H-NMR($CDCl_3$) δ: 0.88 (t, 12H), 1.25 and 1.43 (br.s., 128H), 2.42 (t, 4H), 2.62 (t, 4H), 3.48 (t, 4H), 3.7, (t,4H);

4b. N,N,N',N'-didecyl-2,2'-oxobis ethylamine (93% yield), H-NMR ($CDCL_3$) δ: 0.88 (t,12H), 1.25 and 1.42 (br.s, 64H), 2.42 (t, 8H), 2.63 (t, 4H), 3.5 (t, 4H);

4c. N,N',-octadecyl-2,2'-oxobis ethylamine (71% yield), H-NMR ($CDCl_3$) δ: 0.87 (t, 6H), 1.25 and 1.45 (br.s., 64H), 2.6 (t,4H), 2.78 (t, 4H), 3.65 (t,4H);

4d. N,N,N',N'-octadecylaminopropyl-2,2'-oxobis ethylamine (80% yield), H-NMR (CDCL3) δ:0.88 (t,6H), 1.25 and 1.45 (br.s,70H), 2.4-2.8 (br.m,16H), 3.4-3.7 (br.m, 4H).

Example 4: Synthesis of N,N,N,N',N',N'_- dioctadecylmethyl-2,2'-oxybisethylammonium iodide (5a).

N,N,N',N'-dioctadecyl-2,2'-oxy bis ethylamine (19mg, 0.017mmol) was dissolved in iodo methane (1ml) inside a capped thick-wall test tube, and the resulting solution heated for 20h at 75°C. TLC (silicagel; chloroform:acetone:methanol:water; 50:15:5:5:1) shows only one spot at Rf = 0.8 ,which gives a negative ninhydrin test and no starting material. The excess iodo methane was removed in vacuo to afford desired product (23 mg, 96%). H-NMR($CDCl_3$) δ: 0.88 (t,12H), 1.15 - 1.5 and1.7 (br.s.,128H), 3.35 (s, 6H),

3.46 (br.m.,8H), 3.88 (br.s.,4H), 4.28 (br.s., 4H). Proceeding in a similar fashion as per compound (5a) , compound (5b) was obtained in 100% yield.

Example 5: Synthesis of N,N,N',N'-cyanoethyloctadecyl-2,2'-oxybis ethylamine (4e).

A suspension of N,N'-octadecyl-2,2'-oxybis ethylamine (100mg,0.16mmol) in acrylonitrile (4ml) was heated for 18h at 80°C in a capped, thick-wall test tube (the initially liquid two phase system became a clear homogenous solution after 2h). TLC (silicagel ; dichloromethane / THF, 3:1) shows absence of starting material and a spot corresponding to desired material at rf = 0.95. The excess acrylonitrile was removed in vacuo to afford desired material. H-NMR($CDCl_3$) δ: 0.88 (t, 6H), 1.25 (br.s., 64H), 2.45 (2t,8H), 2.68 (t, 4H), 2.87 (t,4H), 3.5 (t, 4H).

Example 6: Synthesis of N,N,N,N',N',N'-cyanoethyloctadecylmethyl-2,2'-oxybis ethyl ammonium iodide (5e).

A solution of N,N,N',N'-cyanoethyloctadecyl-2,2'-oxybis ethylamine (60 mg,0.08 mmol) in iodo methane (1.5ml) was heated for 3h at 80°C in a capped, thick wall tested tube. The excess iodo methane was removed in vacuo to afford desired product. H-NMR ($CDCl_3$) δ: 0.88 (t, 6H), 1.25 (br.S., 60H), 1.8 (br.s., 4H), 3.45 (br.s., 6H), 3.65 (br.s., 4H), 3.95-4.4 (br.m., 16H).

Example 7: Synthesis of N,N,N,N',N',N'- acetoxyethyldioctadecyl-2,2'-oxybis ethyl ammonium iodide(5f).

A solution of N,N,N',N'-dioctadecyl-2,2'- oxybis ethylamine (16mg, 14.3 mmol) and ethyl iodoacetate (0.5ml) in chloroform (1ml) was heated for 18h at 75 °C in a capped thick wall test tube. The chloroform was removed in vacuo and the residue dissolved in tetrahydrofurane (10ml). To this solution was added thiourea and the mixture stirred at room temperature until no more thiourea went into solution (stoichiometric excess after all iodide is converted to the isothiouronium salt). The mixture was heated for 2h at 70°C and the excess solvent removed in vacuo. The residue was then redissolved in

dichloromethane (10ml) and the solution washed with water (4x 5 ml), dried (Na_2SO_4) , filtered and the solvent removed in vacuo to afford 10mg of desired product.

Example 8: Synthesis of S-methylisothiouronium hydroiodide.

A solution of thiourea (0.8g,10.5mmol) and iodo methane (8.8g,62mmol) in methanol (25ml) was heated in a capped thick-wall test tube for 4.5h at 50°C. The reaction mixture was rotaevaporated to afford pure desired material in 100% yield.H-NMR (CD_3OD) δ: 2.62(s, 3H), 4.8 (br.s., 4H).

Example 9: Synthesis of N,N,N',N'-guanidinopropyloctadecyl oxy bis-2,2'-ethylamine hydroiodide (6d).

A solution of N,N,N',N'-aminopropyloctadecyl-2,2'- oxybis ethylamine (100mg, 0.14 mmol) , S- methyl isothiouronium hydroiodide (300mg, 1.3 mmol) and triethylamine (300mg, 3 mmol) in tetrahydrofurane (10ml) were heated in an argon flushed, capped thick-wall test tube for 20h at 95°C. The solvent and methyl mercaptan by product were removed in vacuo in a chemical fumes hood and the residue dissolved in methylene chloride (30ml), the organic phase was washed with brine (3x, 20 ml), water (2x 10ml), dried (Na_2SO_4), filtered and the solvent removed to obtain a reddish solid (100mg,80%). H-NMR($CDCl_3$) δ: 0.86 (t,6H), 1.1-1.6 (br.s., 64H), 2.4-2.8 (br.m., 16H), 3.15-3.7 (br.m., 12H). FTIR (cm^{-1}): 1653 (C=NH).

Example 10: Synthesis of N,N'-cyanoethyl-1,4-diaminobutane (7).

1,4-diaminobutane (2g, 22 mmol) was cooled to 0C° (ice bath) and to this solid was added acrylonitrile (4ml) . The mixture was let reach room temperature slowly (ca 30min) and then let react for additional 18h at room temperature with stirring. The excess acrylonitrile was removed in vacuo to afford the desired product. H-NMR ($CDCl_3$) δ: 1.3 (br.s.,2H), 1.5 (br.s, 4H), 2.5 (t, 4H), 2.65 (br.m.,4H), 3.9 (t,4H). FTIR (cm^{-1}) : 2247 (C=N).

Example 11: Synthesis of N,N,N',N'-cyanoethylpamitoyl-1,4-diaminobutane(8).

To a solution of N,N'-cyanoethyl-1,4-diaminobutane (0.714g , 3.68mmol) and triethylamine (0.744g ,7.36mmol) in dichloromethane (150ml) was slowly added palmitoyl chloride (2.02g, 7.36 mmol) and the resulting mixture let react at room temperature overnight. The reaction was washed with sodium bicarbonate (10%,2x 50ml), water (2x 50ml), dried (Na_2SO_4) , filtered and the solvent removed in vacuo to afford the desired product (2.3g, 93 %). H-NMR ($CDCL_3$) δ: 1.9 (t,6H), 1.25 and 1.6 (br.s., 56H), 2.32 (m,4H), 2.72 (t, 4H), 3.42 (m, 4H), 3.55 (t,4H). FTIR (cm^{-1}) 1643 (C=O).

Example 12: Synthesis of N,N,N',N'-aminopropylpalmyl-1,4-diaminobutane (9).

To a solution of lithium aluminum hydride (600mg,15.9 mmol) in tetrahydrofurane (50ml) was added N,N,N',N'-cyanoethylpalmitoyl-1,4-diaminobutane (300mg, 0.45 mmol) and the reaction mixture was refluxed for 72 h. Then, a procedure essentially the same as per example 3 (supra) was followed to afford desired diamine (200mg,70%). H-NMR($CDCl_3$) δ: 0.88 (t,6H), 1.25 and 1.5 (br.s, 64H), 2.3-2.7 (br.m, overlap. t, 16H).

Example 13: Synthesis of N,N,N',N'-guanidinopropylpalmyl-1,4-diaminobutane (10).

A procedure identical as per example 6 (supra) was followed. H-NMR ($CDCl_3$) δ: 0.9 (t, 6H), 1.2-1.35 (br.s., 68H), 1.5-2.0 (br.m., 4H), 2.65-3.5 (m, 24H). FTIR (cm^{-1}) : 1650 (C=N).

Example 14: Synthesis of N,N'''-4-bromobutyryl-N,N',N''N'''-tetrapamylspermine (12).

To a cooled (0 ^{0}C) solution of N,N',N'',N'''-tetrapalmylspermine (400mg, 0.36 mmol) and triethylamine (80mg, 0.8 mmol) in dichloromethane (14ml) was added 4-bromobutyryl chloride (156mg, 0.8 mmol) and the resulting mixture let react for 1h at 0 ^{o}C. The reaction was quenched and washed with cold sodium bicarbonate solution (10 % in water,3x 5ml), dried (sodium sulfate), filtered and the solvent removed at room temperature in vacuo to afford a white foam.. H-NMR ($CDCl_3$) δ: 0.88 (t, 12H), 1.15-1.5 (br.s., 136H), 2.12 (t, 4H), 2.3-2.55 (br.m.,12H), 3.2-3.36 (br.m., 8H), 3.62 (t, 4H).

Example 15: Synthesis of N,N',N",N'"-tetrapalmyltetraacetoxyethylspermine iodide salt(13a):

A solution of tetrapalmylspermine (Haces et al.,PCT Int. Pub. No WO/95/17373), 130mg, mmol) in neat ethyl iodoacetate (1.5ml) was heated to 75 °C for 18h. The reaction was worked up following essentially the same procedure as per example 13 (supra) to afford the desired product.

Example 16: Synthesis of N,N'"-4-bromobutyryl-N,N',N",N'"-tetrapalmyl-N',N"-dimethylspermine(13c).

N,N'" - 4-bromobutyryl-N,N',N",N'"-tetrapalmylspermine (350mg,0.25 mmol) was dissolved in iodo methane (3ml) and the resulting solution let react for 2 days at room temperature . Excess iodo methane was evaporated to afford desired product, which is negative for ninhydrin test. H-NMR ($CDCl_3$) δ: 0.88 (t, 12H), 3.41 (br.s., 6H).

Example 17: Synthesis of N,N'"-4-aminobutyryl-N,N',N",N'"-tetrapalmyl-N',N"-dimethylspermine(13d).

To a solution of N,N'"-4-bromobutyryl-N,N',N",N'"-tetrapalmyl-N',N"-dimethylspermine (100mg,0.05 mmol) in tetrahydrofurane (10ml) was added ammonium hydroxide (20 ml, 28% by weight) and the resulting mixture heated to 70 °C for 2 days in a capped, thick wall reaction tube. The solvent was azeotropically evaporated (ethanol) to afford a brown solid which is strongly positive for ninhydrin test.H-NMR ($CDCl_3$) δ: 0.88 (t,12H), 1.1-1.4 (br.s,130H),1.5-2.2 (br.m,16H), 3.0-3.8 (br.m,10H), 3.4 (br.s, 6H).

Example 18: Synthesis of dioleoylphosphatidyl ethanolguanidine (15).

To a solution of dioleolylphosphatidyl ethanoamine (70mg,0.094 mmol) and triethylamine (1ml) in tetrahydrofurane (10 ml) was added S-methylisothiouronium hydroiodide (70mg, 0.32 mmol) and the resolting solution heated for 18h at 70 °C. The solvent was removed in vacuo and the residue redissolved in dichloromethane (25ml). This solution was washed with water (2 x 10 ml), dried (sodium sulfate), filtered and the solvent evaporated to afford the desired product (40 mg, 47%). H-NMR ($CDCl_3$) δ: 0.88

(t,6H), 1.2-1.44 (br.s, 40H), 2.00 (br.d,10H), 2.29 (t, 4H), 3.19 (q,1H), 3.42 (br.s,1H), 3.85-4.20 (br.m,2H), 4.38 (br.m,1H), 5.3 (br.d, 4H). FTIR (cm^{-1}) : 2361, 1741.

Example 19: Liposomes formulation:

Lipids were formulated by mixing the appropriated molar amounts of the active lipid with dioleoylphophatidyl ethanolamine (DOPE) in dichloromethane and dispersing this mixture in the final amount of water using the solvent vaporization method. (David W. Deamer, in Liposome Technology, vol.I, p-29, CRC press Boca Raton, Fl, 1984).

Cell culture and plasmids

Cell lines were from the American Type Culture Collection (Rockville, Maryland) and were cultured in RPMI1649, 10% FCS, pen/strep . Plasmid pCMV β-gal, which contains the *E. Coli* β-galactosidase (gene) under the control of the powerful cytomegalovirus promoter (McGregor et al. (1989) *Nucleic Acids Res.*, 17,:2365) was purchased from Clontech, Inc. Primary cells were from human tracheal isolates and neonatal foreskin.

Example 20: Transfection of HepG2 and HeLa cells.

Cells were plated in 48-well plates ($1cm^2$) at a concentration of $1x\ 10^5$ cells/well in 0.5 ml of RPMI-1640, 10% FCS, Pen/step. The next day, lipids aliquats (1,3 and 5µl of 1mg/ml liposome in water) were diluted in polystyrene tubes containing 100µl of serum-free, antibiotic-free RPMI-1640 and to these tubes were added 150 ng of plasmid in 100µµl of the same medium (suboptimal amount in polypropylene tubes) and incubate for 15 min. The cells were washed twice with Dulbecco's PBS, the lipid:plasmid complexes added to them and then incubated for 7h at 37 C^o in 5% CO_2 atmosphere. Growth medium was added to the cells for a final volume of 1 ml and a final concentration of 10 % FCS, pen/strep, 50ug/ml gentamicin in RPMI-1640 and were incubated overnight.

Example 21: Transfection of Primary Human Tracheobronchial and Epidermal Keratinocytes.

Cells were grown in serum free medium (SFM) and plated on 35 mm plates (6wells) such that the confluence after 24h was above 50%. Plasmid reporter (2μg and 5μg, respectively) was mixed with variable amounts of liposomes (see tables IV and V) and the complex formed added to the cells. The cells were transfected during 4h and 6h, respectively. The DNA/liposome complex was removed by rinsing with SFM and the cells incubated for 48h under normal growth conditions and then assayed for the appropiate marker.

Example 22: Transfection and CAT assay of Jurkat Cells (suspension cells).

The cell suspension culture was transferred to a 50 ml conical tube and centrifuged at 400g for 10 min. The cells were washed twice by aspirating off the supernatant and gently resuspending the cell pellet in 25 ml of sterile PBS and centrifuging again at 400g for 10min. The pellet was resuspended in a volume of serum-free growth medium such that a final concentration of 6.25×10^6 cells /ml is obtained (about 10 ml). 35 mm cell culture plates were inoculated with 0.8 ml of the cell suspension. For each well, 10 μg of CAT plasmid were dissolved in 110 μl of serum-free medium and a separately in another tube were diluted 30 μl of the lipid solution in 70 μl of serum-free medium. The plasmid and lipid solution were mixed and gently swirled and let stand at room temperature for 10 min. The complex DNA/lipid solution was then randomly dropped over the culture well. The wells were gently swirled and then incubated at 37 ^{0}C under a 7 % CO_2 atmosfere for 5 hours. After 5h incubation, 4ml of 12.5 % FBS growth medium were added to the wells and the incubation continued for additional 72 h under the above conditions. The cells were then transferred to 10 ml Falcon tubes and the wells rinsed with 5ml of sterile PBS . The cell suspension was washed twice with 5ml of sterile PBS as previously. The final pellet was resuspended in 400 μl of lysis buffer and transferred to 1.5 ml centrifugation tubes. The tubes were capped and placed horizontally on a rocker and the cells lysated for 30 min.

100 µl aliquats were then assayed for CAT activity following the procedure of Neumann et al. (1987) *Biotechniques* 5: 444

Example 23: Assay for transient transfection (adherent cells).

The cells were washed twice with Dulbecco's PBS and stained with freshly prepared fixative (2%formaldehyde/ 0.2% glutaraldehyde in PBS) for 5 min, washed twice with Dulbecco PBS.). Then, stained with 0.5 ml of β-galactosidase histochemical stain (0.1% x-gal, 5mM potassium ferrocyanide, 5mM potassium ferrocyanide, 2mM MgCl2 in PBS) for 24h at 37 C^o in a 5% CO_2 atmosphere. Blue cells (β-gal positive) were counted.

Example 24: Results and Discussion.

Results are summarized on tables I , II ,III, IV and V. Tables I and II show the relative transfection efficiency of compounds 5a, 6d and 13d versus control compound TMTPS (Compound 3 in PCT Int.Pub.No. WO95/17373. Haces, A. et al.) in HepG2 (human hepatocarcinoma) and HeLaS3 (human cervical carcinoma) cells, respectively. And under suboptimal conditions for activity. In these cell lines, compounds 6d and 13d show a 2-2.4 fold higher efficiency than the TMTPS control , and compound 5a is half as active as control in HepG2 cells and showed negligible activity in HeLaS3 cells. Table III shows an analogous comparison using Jurkat cells (T-cell leukemia). In this experiment, compounds 5a and 13d show similar efficiency as TMTPS, but compound 6d shows almost 38% more activity than that of the control. Tables IV and V shows the relative efficiency of compounds 6d and 13d in the primary human tracheobronchial epithelial and human keratinocytes cells. Primary cells are cells that are freshly isolated from humans or animals and which , unlike the cultured cell lines, reflect the potential behavior of a compound in vivo more closely. Thus, for genetic therapy to work, it is necessary to be able to transfect these types of cell lines before any in vivo experiments are tried. These types of cells are also the most difficult to transfect and their transfection efficiencies are usually below 1%. Table IV shows the relative efficiency of compounds 6d and 13d

versus DOTMA (Lipofectin™ Reagent, Life Technologies, Inc.,Supra) in primary human Tracheo brochial cells. Both of these compounds show a relative range of activities of 5.3 to 6.0 times higher than that of the Lipofectin control. At the same time their cell toxicity was below 5%, unlike the control which showed toxicity in the 10-20% range. Thus, these lipid reagents are superior to the commercial standards in both respects. In addition, this is a very significant result since tracheobronchial cells are involved in the genetic disease cystic fibrosis. There are several genetic therapy clinical trials being conducted at the present time targeting these cells using either viral or liposomal vectors (see 10th Annual North America Cystic Fibrosis Conference, Orlando, Fl., Oct 24-27 (1996), Abstracts or *Pediatr Pulmonol* Suppl, 13: 74-365, Sept, 1996) .

Table V depicts the the percentages of β-gal positive cells (absolute number) which were obtained in primary human epidermal keratinocytes with compounds 5a and 6d versus that obtained with DOSPA control (Lipofectamine™ Reagent, Life Technologies, Inc. Supra) . Compounds 5a and 6d gave, respectively, 35% and 50% positive cells as compared with 2% positives for the control. This represents a 15-25 fold better efficiency for these novel liposome reagents when compared with this well known standard. Moreover, primary human keratinocytes are also a potential target cells for genetic therapy (Fenjves, E.S. et al., *Hum Gene Ther* 5: 10,1241-8, Oct. 1994.), but its use has been restricted due to the lack of highly efficient transfection vectors.

TABLE I

TRANSFECTION OF HEP G2 CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount(μg)	β- Gal Positive Cells (%)
Compound 5a (1: 1.8)	3 μg	0.8
Compound 6d (1: 1.5)	3 μg	4.3
Compound 13d (1:1.5)	3 μg	2.9
Control TMTPS/DOPE(1:1.5)	5 μg	1.5

Cells were plated in 48 well plates at a density of 1 x 10^5 per well in 0.5 ml of growth medium. After 24h, the cells were washed with serum free medium and transfected with a **suboptimal** amount (150 ng) of plasmid pCMV-βgal. using 1,3 and 5ul (1,3 and 5μg) of lipid formulation. The amount giving the highest level of transfection efficiency is shown. The experiment was run in triplicate.

TABLE II
TRANSFECTION OF He La S3 CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	β-Gal Positive Cells (%)
Compound 5a (1:1.8)	5 μg	0.001
Compound 6d (1:1.5)	3 μg	2.4
Compound 13d (1:1.5)	1 μg	2.1
Control TMTPS/DOPE(1:1.5)	5 μg	0.9

Cells were plated in 48 well plates at a density of 1 x 10^5 per well in 0.5 ml of growth medium. After 24h, the cells were washed with serum free medium and transfected with a **suboptimal** amount (150 ng) of plasmid pCMV-βgal. using 1,3 and 5ul (1,3 and 5μg) of lipid formulation. The amount giving the highest level of transfection efficiency is shown. The experiment was run in triplicate.

TABLE III
TRANSFECTION OF JURKAT CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	CAT Activity (mU/well)
Compound 5a (1:1.8)	30 μg	196.00
Compound 6d (1:1.5)	30 μg	298.20
Compound 13d(1:1.5)	30 μg	220.00
Control TMTPS/DOPE(1:1.5)	30 μg	216.44

Wells were inoculated with 6.25 x 10^6 cells . 10 μg of CAT plasmid were mixed with 30μg (optimal amount known for the control) of the lipids and then added to the cells. After 5h the transfection was quenched with FBS containing medium and the cells incubated for 72h. Cells were lysated in 400 μl of buffer. 100 μl aliquats were assayed for CAT activity.

TABLE IV
TRANSFECTION OF PRIMARY HUMAN TRACHEOBRONCHIAL EPITHELIAL CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	Luciferase Activity (counts)
Compound 6d (1:1.5)	12 μg	7297022
Compound 13d (1:1.5)	12 μg	8343975
Control , Lipofectin™*	6 μg	1379341

35 mm plates were inoculated with cell isolates and transfected at 90% confluence. 2μg of firefly luciferase plasmid were mixed with 12 μg of the lipids and then added to the cells. After 5h, the transfection was quenched by removal of the DNA/Lipid complex and the cells incubated for 72h. Cells were lysated and aliquats assayed for luciferase activity. Cell toxicity , determined by the trypan blue method, was below 5% for lipids 6d and 13d and between 10-20% for Lipofectin.* Lipofectamine was also run as a control, but its efficiency was negligible.

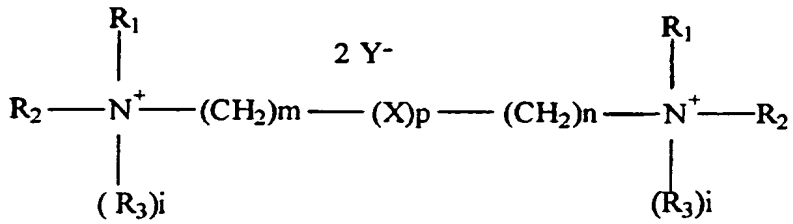
TABLE V
TRANSFECTION OF PRIMARY HUMAN EPIDERMAL KERATINOCYTES

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	β-Gal Positive Cells (%)
Compound 5a (1:1.5)	40 μg	35 %
Compound 6d (1:1.5)	20 μg	50 %
Control , Lipofectamine™	25 μg	2 %

Cells were seeded at $2x10^5$ /well in 35 mm wells and transfected the next day. 5μg of βgal DNA were mixed with the appropiate amount of lipids and added to the cells. After 4h, the medium was replaced and the cells incubated for additional 48h and then assayed. Blue cells were observed under the microscope and counted.

What is Claimed:

1. A composition comprising the mixture of a compound having the structure:



Y= Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion

X = O,S(O),CH_2 R_1,R_1' = independently: C_1-C_{18} linear hydrocarbon.

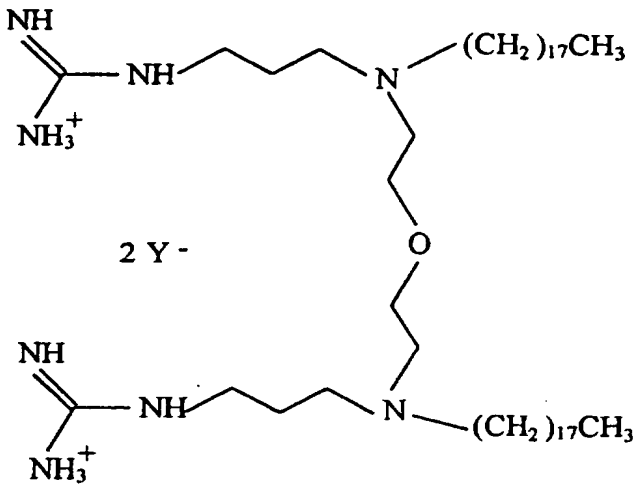
m,n = 1,2,3 R_2,R_2' = independently : H ; C_1-C_{18} linear alkyl; cyanoethyl; aminopropyl; aminobutyl; C_2 -C_4 alkyl guanidinium or amidinium; N,N,N- independently (C_1-C_{18})aminopropyl or aminobutyl; C or N substituted spermine or spermidine; N,N-(C_4-C_{18})alkyl-4- aminonobutyrylaminopropyl

p = 0, 1 R_3,R_3' = independently: C_1-C_6 linear alkyl, acetoxyethyl,
i = 0, 1 $CH_2CO_2CH_2CH_3$

$X \neq CH_2$ when R_2,R_2' = C_1-C_6 linear alkyl
When p = 1, m and n ≠ 1

and a member of the class consisting of nucleic acids, oligonucleotides, mononucleotides, polypeptides and proteins.

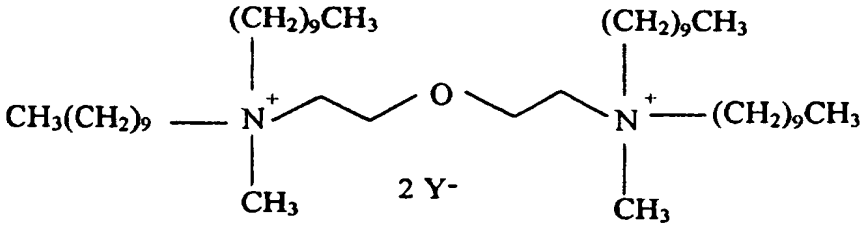
2. A composition comprising the mixture of a compound having the structure:



Y = Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

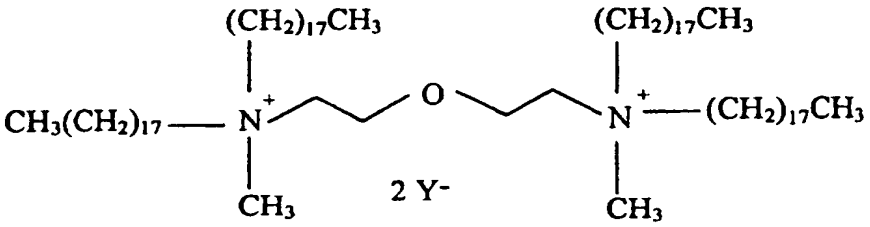
3. A composition comprising the mixture of a compound having the structure:



Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

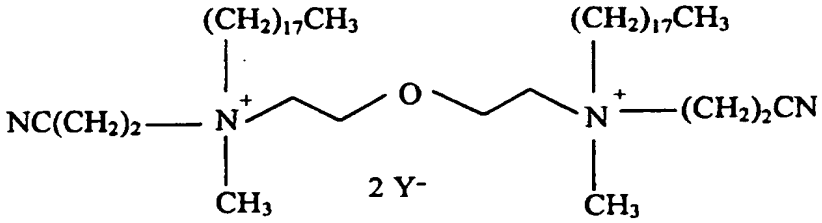
4. A composition comprising the mixture of a compound having the structure:



Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

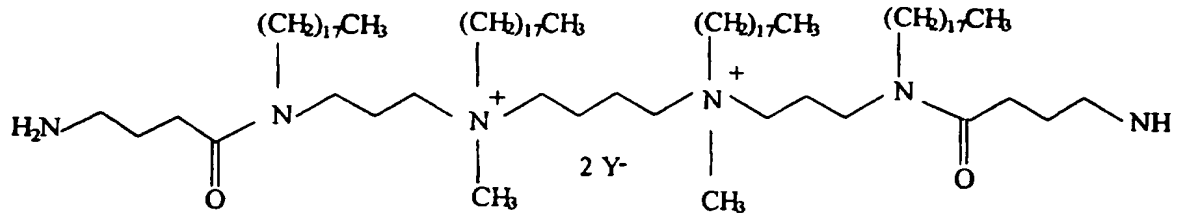
5. A composition comprising the mixture of a compound having the structure:



Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

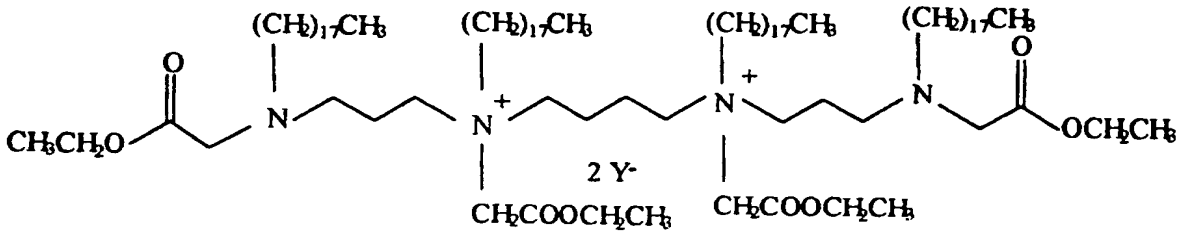
6. A composition comprising the mixture of a compound having the structure:



Y = Cl, Br, I, AcO, or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

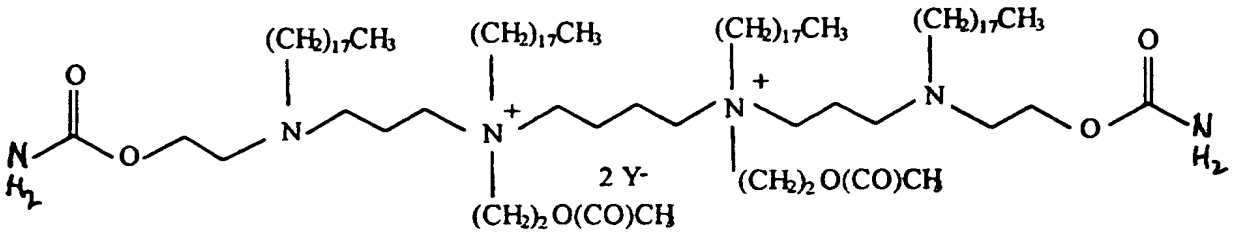
7. A composition comprising the mixture of a compound having the structure:



Y = Cl, Br, I, AcO, or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids, oligonucleotides,mononucleotides, polypeptides and proteins.

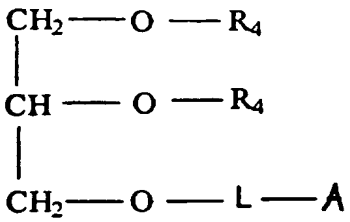
8. A composition comprising the mixture of a compound having the structure:



Y = Cl-, Br-, I-, AcO-, or any pharmaceutically acceptable anion.

and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

9. A composition comprising the mixture of a compound having the structure:



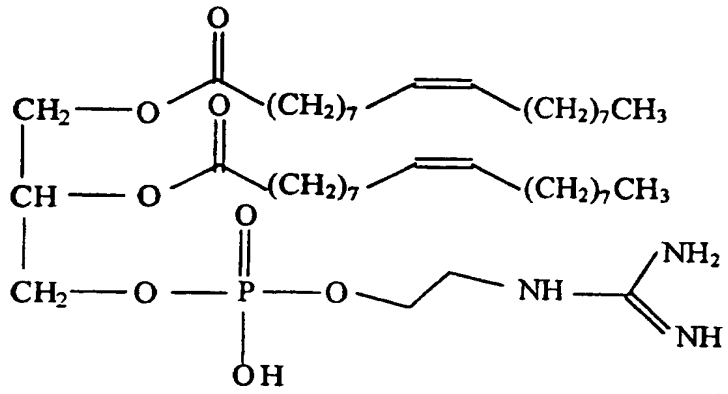
R_4 = Independently: linear $(CO)C_6$-C_{20}, C_6-C_{20}

L = 2-10 atoms linker

A = Guanidinium; imidinium; guanidylated polyamines.

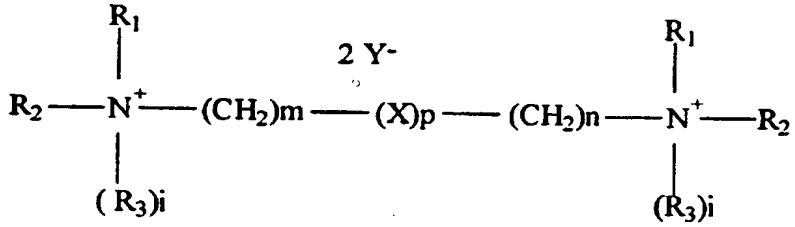
and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

10. A composition comprising the mixture of a compound having the structure:



and a member of the class consisting of nucleic acids,oligonucleotides,mononucleotides, polypeptides and proteins.

11. A compound having the formula:



Y= Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion

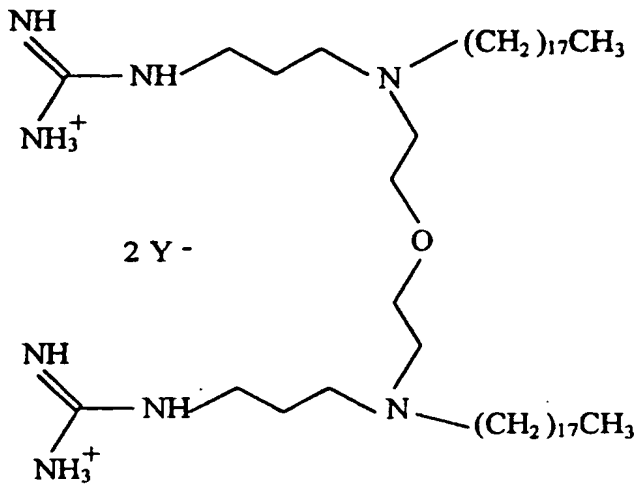
X = O,S(O),CH_2 R_1,R_1' = independently: C_1-C_{18} linear hydrocarbon.

m,n = 1,2,3 R_2,R_2' = independently : H ; C_1-C_{18} linear alkyl; cyanoethyl; aminopropyl; aminobutyl; C_2 -C_4 alkyl guanidinium or amidinium; N,N,N- independently (C_1-C_{18})aminopropyl or aminobutyl; C or N substituted spermine or spermidine; N,N-(C_4-C_{18})alkyl-4- aminonobutyrylaminopropyl

p = 0, 1 R_3,R_3' = independently: C_1-C_6 linear alkyl, acetoxyethyl,
i = 0, 1 $CH_2CO_2CH_2CH_3$

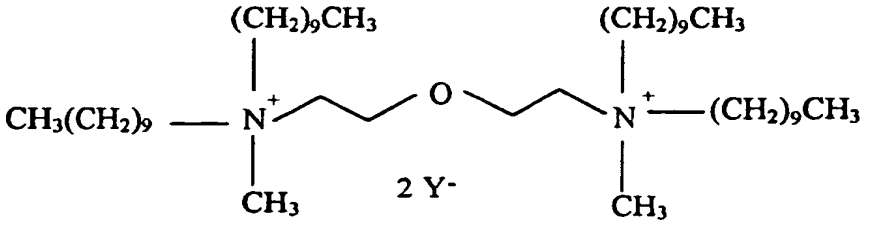
X ≠CH_2 when R_2,R_2' = C_1-C_6 linear alkyl
When p = 1, m and n ≠1

12. A compound having the structure:



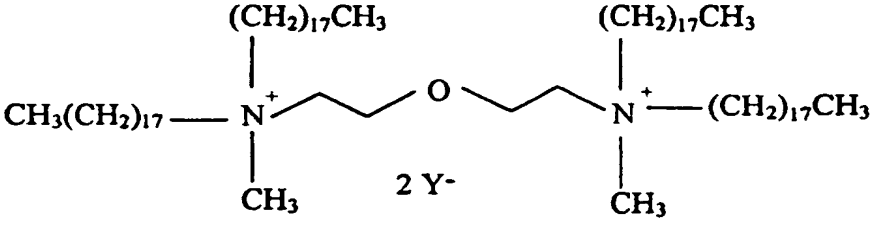
Y = Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion.

13. A compound having the structure:



Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion.

14. A compound having the structure:



Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion.

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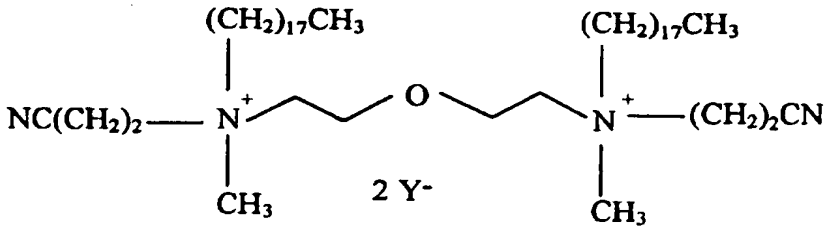
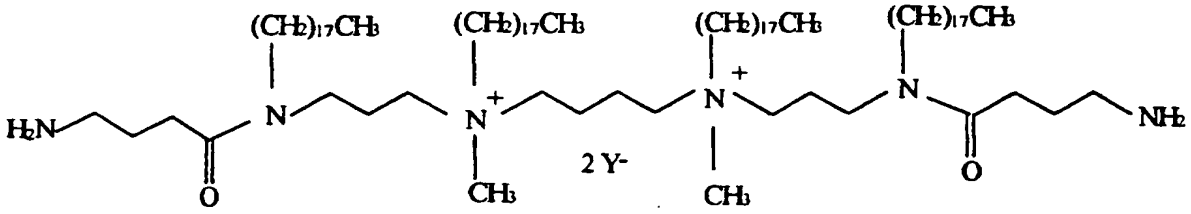


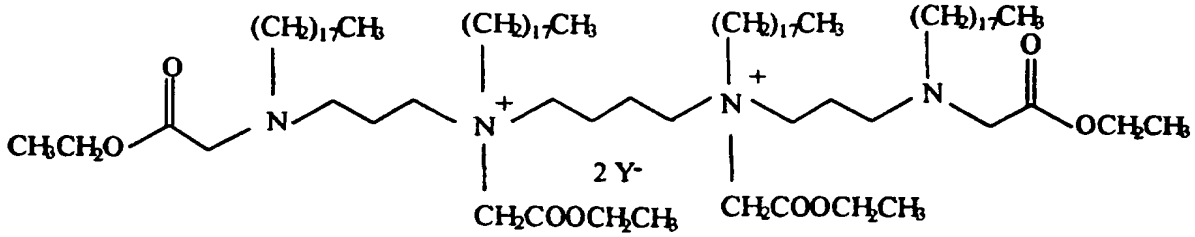
Y = I^-, Br^-, Cl^-, AcO^- or any pharmaceutically acceptable anion

16. A compound having the formula:



Y = Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion.

17. A compound having the structure:



Y = Cl^-, Br^-, I^-, AcO^-, or any pharmaceutically acceptable anion.

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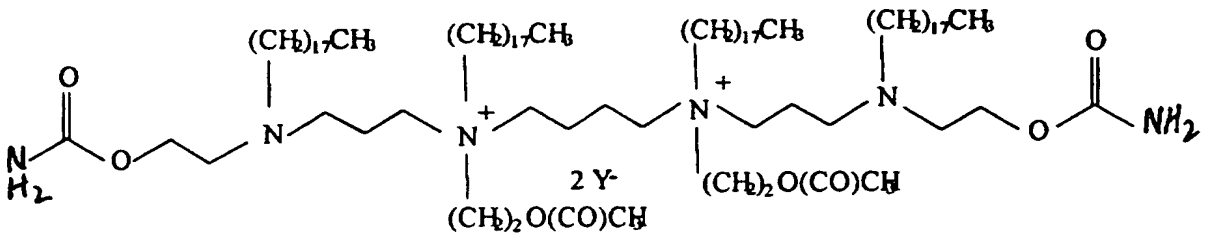


Y = Cl, Br,I-, AcO, or any pharmaceutically acceptable anion.

19. A compound having the structure:

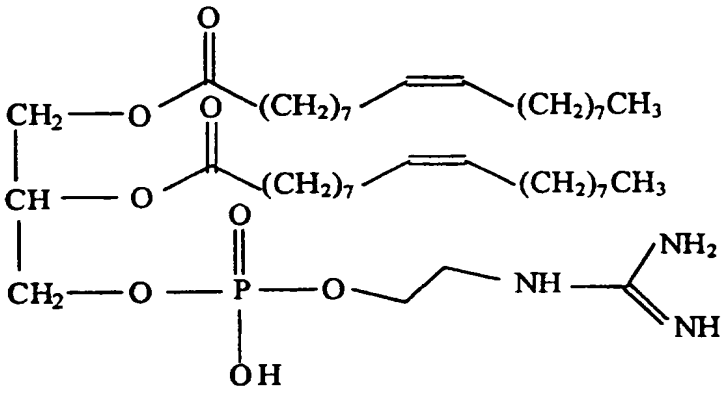
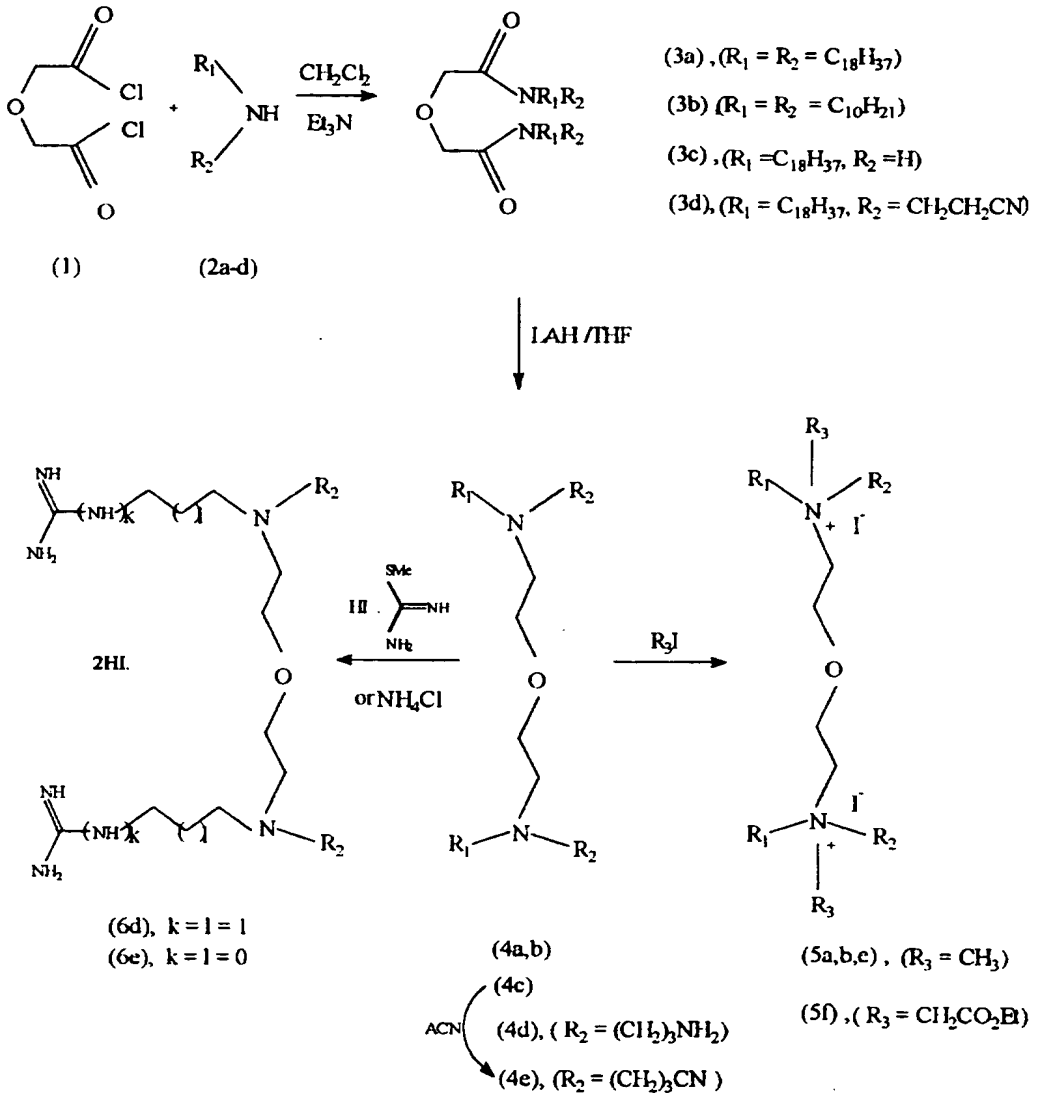


FIG.1



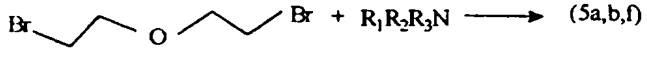


FIG. 2

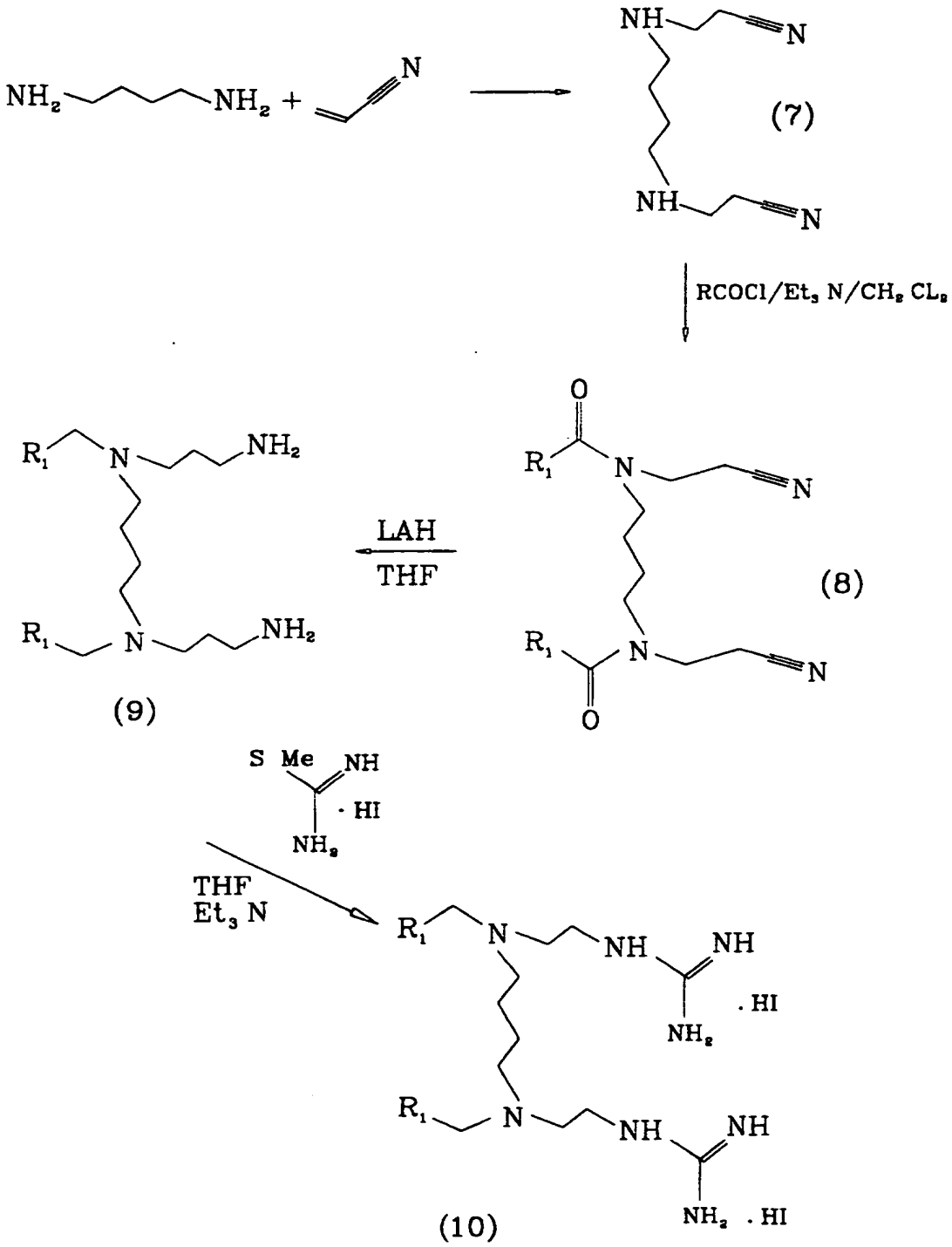


FIG. 3

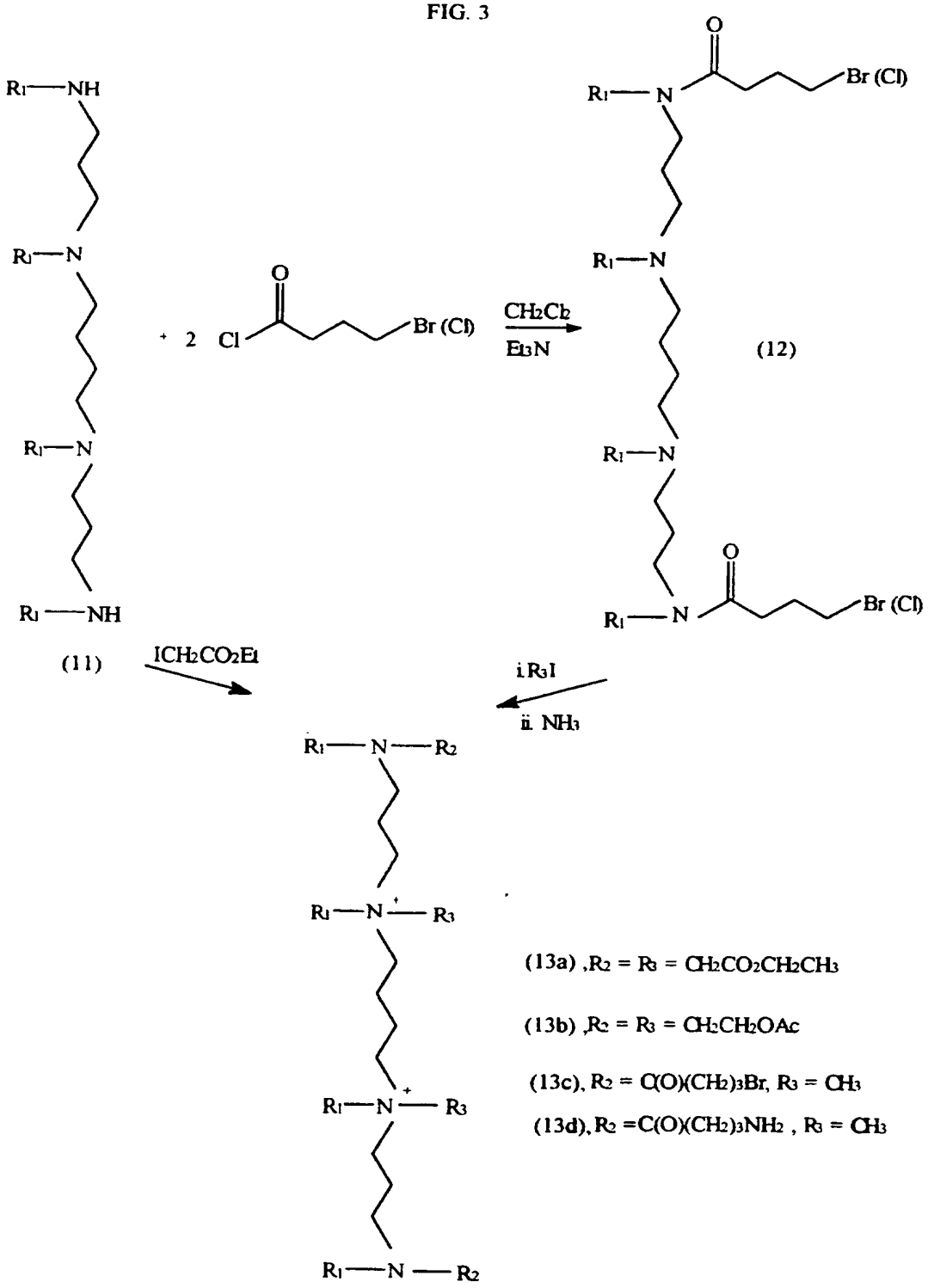
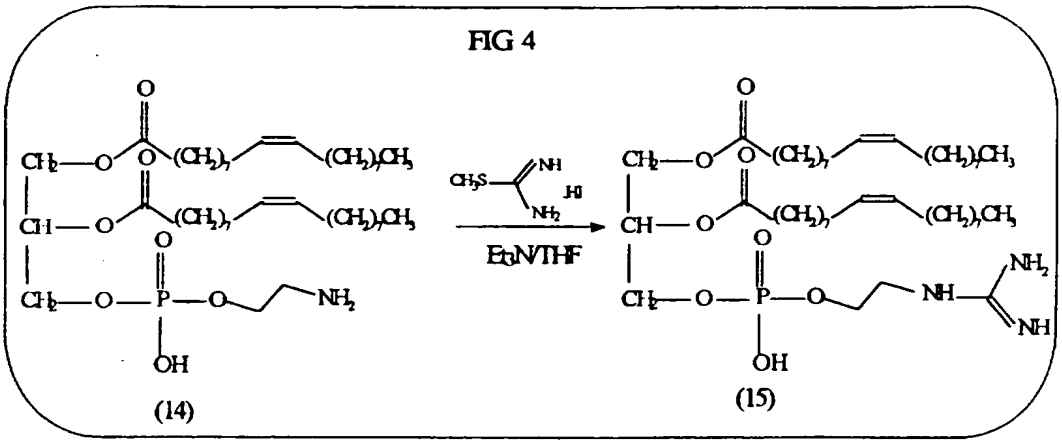


FIG 4



(14) (15)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09093

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 37/18; A61K 37/16
US CL :514/2,7
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2,7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG: CAS, Medline, Derwent Biotechnology Abstracts, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,459,127 A (FLEGNER et al.) 17 October 1995, see entire document.	1-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
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